SYNTHETIC DNA SEQUENCE HAVING ENHANCED INSECTICIDAL ACTIVITY IN MAIZE

This is a continuation of U.S. application no. 09/547,422, filed April 11, 2000, which is a continuation of U.S. application no. 08/459,504, filed June 2, 1995, now U.S. Patent No. 6,075,185, which is a division of U.S. application no. 07/951,715, filed September 25, 1992, now U.S. Patent No. 5,625,136, which is a continuation-in-part of U.S. application no. 07/772,027, filed October 4, 1991, now abandoned, which disclosures are herein incorporated in their entireties.

FIELD OF THE INVENTION

The present invention relates to DNA sequences encoding insecticidal proteins, and expression of these sequences in plants.

BACKGROUND OF THE INVENTION

Expression of the insecticidal protein (IP) genes derived from *Bacillus thuringiensis* (Bt) in plants has proven extremely difficult. Attempts have been made to express chimeric promoter/Bt IP gene combinations in plants. Typically, only low levels of protein have been obtained in transgenic plants. See, for example, Vaeck et al., Nature 328:33-37, 1987; Barton et al., Plant Physiol. 85:1103-1109, 1987; Fischoff et al., Bio/Technology 5:807-813, 1987.

One postulated explanation for the cause of low expression is that fortuitious transcription processing sites produce aberrant forms of Bt IP mRNA transcript. These aberrantly processed transcripts are non-functional in a plant, in terms of producing an insecticidal protein. Possible processing sites include polyadenylation sites, intron splicing sites, transcriptional termination signals and transport signals. Most genes do not contain sites that will deleteriously affect gene expression in that gene's normal host organism. However, the fortuitous occurrence of such processing sites in a coding region might complicate the expression of that gene in transgenic hosts. For example, the coding region for the Bt insecticidal crystal protein gene derived from *Bacillus thuringiensis* strain *kurstaki* (GENBANK BTHKURHD, accession M15271, *B. thuringiensis* var. *kurstaki*, HD-1; Geiser et al. Gene 48:109-118 (1986))

as derived directly from *Bacillus thuringiensis*, might contain sites which prevent this gene from being properly processed in plants.

Further difficulties exist when attempting to express *Bacillus thuringiensis* protein in an organism such as a plant. It has been discovered that the codon usage of a native Bt IP gene is significantly different from that which is typical of a plant gene. In particular, the codon usage of a native Bt IP gene is very different from that of a maize gene. As a result, the mRNA from this gene may not be efficiently utilized. Codon usage might influence the expression of genes at the level of translation or transcription or mRNA processing. To optimize an insecticidal gene for expression in plants, attempts have been made to alter the gene to resemble, as much as possible, genes naturally contained within the host plant to be transformed.

Adang et al., EP 0359472 (1990), relates to a synthetic *Bacillus thuringiensis tenebrionis* (Btt) gene which is 85% homologous to the native Btt gene and which is designed to have an A+T content approximating that found in plants in general. Table 1 of Adang et al. show the codon sequence of a synthetic Btt gene which was made to resemble more closely the normal codon distribution of dicot genes. Adang et al. state that a synthetic gene coding for IP can be optimized for enhanced expression in monocot plants through similar methods, presenting the frequency of codon usage of highly expressed monocot proteins in Table 1. At page 9, Adang et al. state that the synthetic Btt gene is designed to have an A+T content of 55% (and, by implication, a G+C content of 45%). At page 20, Adang et al. disclose that the synthetic gene is designed by altering individual amino acid codons in the native Bt gene to reflect the overall distribution of codons preferred by dicot genes for each amino acid within the coding region of the gene. Adang et al. further state that only some of the native Btt gene codons will be replaced by the most preferred plant codon for each amino acid, such that the overall distribution of codons used in dicot proteins is preserved.

Fischhoff et al., EP 0 385 962 (1990), relates to plant genes encoding the crystal protein toxin of *Bacillus thuringiensis*. At table V, Fischhoff et al. disclose percent usages for codons for each amino acid. At page 8, Fischoff et al. suggest modifying a native Bt gene by removal of putative polyadenylation signals and ATTTA sequences. Fischoff et al. further suggest scanning the native Bt gene sequence for regions with greater than four consecutive adenine or thymine nucleotides to identify putative plant polyadenylation signals. Fischoff et al.

state that the nucleotide sequence should be altered if more than one putative polyadenylation signal is identified within ten nucleotides of each other. At page 9, Fischoff et al. state that efforts should be made to select codons to preferably adjust the G+C content to about 50%.

Perlak et al., PNAS USA, 88:3324-3328 (1991), relates to modified coding sequences of the *Bacillus thuringiensis* cryIA(b) gene, similar to those shown in Fischoff et al. As shown in table 1 at page 3325, the partially modified cryIA(b) gene of Perlak et al. is approximately 96% homologous to the native cryIA(b) gene (1681 of 1743 nucleotides), with a G+C content of 41%, number of plant polyadenylation signal sequences (PPSS) reduced from 18 to 7 and number of ATTTA sequences reduced from 13 to 7. The fully modified cryIA(b) gene of Perlak et al. is disclosed to be fully synthetic (page 3325, column 1). This gene is approximately 79% homologous to the native cryIA(b) gene (1455 of 1845 nucleotides), with a G+C content of 49%, number of plant polyadenylation signal sequences (PPSS) reduced to 1 and all ATTTA sequences removed.

Barton et al., EP 0431 829 (1991), relates to the expression of insecticidal toxins in plants. At column 10, Barton et al. describe the construction of a synthetic AaIT insect toxin gene encoding a scorpion toxin using the most preferred codon for each amino acid according to the chart shown in Figure 1 of the document.

SUMMARY OF THE INVENTION

The present invention is drawn to methods for enhancing expression of heterologous genes in plant cells. Generally, a gene or coding region of interest is constructed to provide a plant specific preferred codon sequence. In this manner, codon usage for a particular protein is altered to increase expression in a particular plant. Such plant optimized coding sequences can be operably linked to promoters capable of directing expression of the coding sequence in a plant cell.

Specifically, it is one of the objects of the present invention to provide synthetic insecticidal protein genes which have been optimized for expression in plants.

It is another object of the present invention to provide synthetic Bt insecticidal protein genes to maximize the expression of Bt proteins in a plant, preferably in a maize plant. It is one feature of the present invention that a synthetic Bt IP gene is constructed using the most preferred

maize codons, except for alterations necessary to provide ligation sites for construction of the full synthetic gene.

According to the above objects, we have synthesized Bt insecticidal crystal protein genes in which the codon usage has been altered in order to increase expression in plants, particularly maize. However, rather than alter the codon usage to resemble a maize gene in terms of overall codon distribution, we have optimized the codon usage by using the codons which are most preferred in maize (maize preferred codons) in the synthesis of the synthetic gene. The optimized maize preferred codon usage is effective for expression of high levels of the Bt insecticidal protein. This might be the result of maximizing the amount of Bt insecticidal protein translated from a given population of messenger RNAs. The synthesis of a Bt IP gene using maize preferred codons also tends to eliminate fortuitous processing sites that might occur in the native coding sequence. The expression of this synthetic gene is significantly higher in maize cells than that of the native IP Bt gene.

Preferred synthetic, maize optimized DNA sequences of the present invention derive from the protein encoded by the cryIA(b) gene in *Bacillus thuringiensis* var. kurstaki, HD-1: Geiser et al., Gene, 48:109-118 (1986) or the cryIB gene (AKA Crya4 gene) described by Brizzard and Whiteley, Nuc. Acids. Res., 16:2723 (1988). The DNA sequence of the native kurstaki HD-1 cryIA(b) gene is shown as SEQ ID NO:1. These proteins are active against various lepidopteran insects, including *Ostrinia nubilalis*, the European Corn Borer.

While the present invention has been exemplified by the synthesis of maize optimized Bt protein genes, it is recognized that the method can be utilized to optimize expression of any protein in plants.

The instant optimized genes can be fused with a variety of promoters, including constitutive, inducible, temporally regulated, developmentally regulated, tissue-preferred and tissue-specific promoters to prepare recombinant DNA molecules, i.e., chimeric genes. The maize optimized gene (coding sequence) provides substantially higher levels of expression in a transformed plant, when compared with a non-maize optimized gene. Accordingly, plants resistant to Coleopteran or Lepidopteran pests, such as European corn borer and sugarcane borer, can be produced.

It is another object of the present invention to provide tissue-preferred and tissue-specific promoters which drive the expression of an operatively associated structural gene of interest in a specific part or parts of a plant to the substantial exclusion of other parts.

It is another object of the present invention to provide pith-preferred promoters. By "pith-preferred," it is intended that the promoter is capable of directing the expression of an operatively associated structural gene in greater abundance in the pith of a plant than in the roots, outer sheath, and brace roots, and with substantially no expression in seed.

It is yet another object of this invention to provide pollen-specific promoters. By "pollen-specific," it is intended that the promoter is capable of directing the expression of an operatively associated structural gene of interest substantially exclusively in the pollen of a plant, with negligible expression in any other plant part. By "negligible," it is meant functionally insignificant.

It is yet another object of the present invention to provide recombinant DNA molecules comprising a tissue-preferred promoter or tissue-specific promoter operably associated or linked to a structural gene of interest, particularly a structural gene encoding an insecticidal protein, and expression of the recombinant molecule in a plant.

It is a further object of the present invention to provide transgenic plants which express at least one structural gene of interest operatively in a tissue-preferred or tissue-specific expression pattern.

In one specific embodiment of the invention disclosed and claimed herein, the tissue-preferred or tissue-specific promoter is operably linked to a structural gene encoding an insecticidal protein, and a plant is stably transformed with at least one such recombinant molecule. The resultant plant will be resistant to particular insects which feed on those parts of the plant in which the gene(s) is(are) expressed. Preferred structural genes encode B.t. insecticidal proteins. More preferred are maize optimized B.t. IP genes.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a comparison of the full-length native Bt cryIA(b) gene (BTHKURHD; SEQ ID NO:1), a full-length synthetic maize optimized Bt cryIA(b) gene (flsynbt.fin; SEQ ID NO:4) and a truncated synthetic maize optimized Bt cryIA(b) gene (bssyn; SEQ ID NO:3). This figure

shows that the full-length synthetic maize optimized cryIA(b) gene sequence matches that of the native cryIA(b) gene at about 2354 out of 3468 nucleotides (approximately 68% homology).

Fig. 2 is a comparison of the truncated native Bt cryIA(b) gene (nucleotides 1 to 1947 of BTHKURHD; SEQ ID NO:1) and a truncated synthetic maize optimized Bt gene (bssyn; SEQ ID NO:3). This figure shows that the truncated synthetic maize optimized cryIA(b) gene sequence matches that of the native cryIA(b) gene at about 1278 out of 1947 nucleotides (approximately 66% homology).

Fig. 3 is a comparison of the pure maize optimized Bt gene sequence (syn1T.mze; SEQ ID NO:2) with a truncated synthetic maize optimized Bt gene (bssyn; SEQ ID NO:3) and a full-length synthetic maize optimized Bt gene modified to include restriction sites for facilitating construction of the gene (synful.mod; SEQ ID NO:4). This figure shows that the truncated synthetic maize optimized cryIA(b) gene sequence matches that of the pure maize optimized cryIA(b) gene at 1913 out of 1947 nucleotides (approximately 98% homology).

Fig. 4 is a comparison of a native truncated Bt cryIA(b) gene (nucleotides 1 to 1845 of BTHKURHD SEQ ID NO:1) with a truncated synthetic cryIA(b) gene described in Perlak et al., PNAS USA, 88:3324-3328 (1991) (PMONBT SEQ ID NO:5) and a truncated synthetic maize optimized Bt gene (bssyn SEQ ID NO:3). This figure shows that the PMONBT gene sequence matches that of the native cryIA(b) gene at about 1453 out of 1845 nucleotides (approximately 79% homology), while the truncated synthetic maize optimized Bt cryIA(b) gene matches the native cryIA(b) gene at about 1209 out of 1845 nucleotides (approximately 66% homology).

Fig. 5 is a comparison of a truncated synthetic cryIA(b) gene described in Perlak et al., PNAS USA, 88:3324-3328 (1991) (PMONBT SEQ ID NO:5) and a truncated synthetic maize optimized Bt cryIA(b) gene (bssyn SEQ ID NO:3). This figure shows that the PMONBT gene sequence matches that of the truncated synthetic maize optimized Bt cryIA(b) gene at about 1410 out of 1845 nucleotides (approximately 77% homology).

Fig. 6 is a full-length, maize optimized CryIB gene (SEQ ID NO:6) encoding the CryIB protein (SEQ ID NO:7).

Fig. 7 is a full-length, hybrid, partially maize optimized DNA sequence of a CryIA(b) gene (SEQ ID NO:8) which is contained in pCIB4434. The synthetic region is from nucleotides 1-1938 (amino acids 1-646 SEQ ID NO:9), and the native region is from nucleotides 1939-3468

(amino acids 647-1155 SEQ ID NO:9). The fusion point between the synthetic and native coding sequences is indicated by a slash (/) in the sequence.

Fig. 8 is a map of pCIB4434.

Fig. 9 is a full-length, hybrid, maize optimized DNA sequence (SEQ ID NO:10) encoding a heat stable CryIA(b) protein (SEQ ID NO:11), contained in pCIB5511.

Fig. 10 is a map of pCIB5511.

Fig. 11 is a full-length, hybrid, maize optimized DNA sequence (SEQ ID NO:12) encoding a heat stable CryIA(b) protein (SEQ ID NO:13), contained in pCIB5512.

Fig. 12 is a map of pCIB5512.

Fig. 13 is a full-length, maize optimized DNA sequence (SEQ ID NO:14) encoding a heat stable CryIA(b) protein (SEQ ID NO:15), contained in pCIB5513.

Fig. 14 is a map of pCIB5513.

Fig. 15 is a full-length, maize optimized DNA sequence (SEQ ID NO:16) encoding a heat-stable CryIA(b) protein (SEQ ID NO:17), contained in pCIB5514.

Fig. 16 is a map of pCIB5514.

Fig. 17 is a map of pCIB4418.

Fig. 18 is a map of pCIB4420.

Fig. 19 is a map of pCIB4429.

Fig. 20 is a map of pCIB4431.

Fig. 21 is a map of pCIB4428.

Fig. 22 is a map of pCIB4430.

Fig. 23A is a table containing data of cryIA(b) protein levels in transgenic maize.

Fig. 23B is a table which summarizes results of bioassays of *Ostrinia* and *Diatraea* on leaf material from maize progeny containing a maize optimized CryIA(b) gene.

Fig. 23C is a table containing data of cryIA(b) protein levels in transgenic maize.

Fig. 23D is a table which summarizes the results of bioassays of *Ostrinia* and *Diatraea* on leaf material from maize progeny containing a synthetic Bt. maize gene operably linked to a pith promoter.

Fig. 23E is a table containing data on expression of the cryIA(b) gene in transgenic maize using the pith-preferred promoter. Leaf samples from small plantlets transformed with

pCIB4433 using procedures described elsewhere were analyzed for the presence of the cryIA(b) protein using ELISA. All plants expressing cryIA(b) were found to be insecticidal in the standard European corn borer bioassay. Note that the pith-preferred promoter has a low, but detectable level of expression in leaf tissue of maize. Detection of CryIA(b) protein is consistent with this pattern of expression.

Fig. 24 is a complete genomic DNA sequence (SEQ ID NO:18) encoding a maize tryptophan synthase-alpha subunit (TrpA) protein (SEQ ID NO:19). Introns, exons, transcription and translation starts, start and stop of cDNA are shown. $ = start and end of cDNA; +1 = transcription start; 73******* = primer extension primer; +1 = start of translation; +++ = stop codon; bp 1495-99 = CCAAT Box; bp 1593-1598 = TATAA Box; bp 3720-3725 = poly A addition site;

above underlined sequences are PCR primers.

Figs. 25A, 25B, 25C and 25D are Northern blot analyses which show differential expression of the maize TrpA subunit gene in maize tissue at 2 hour, 4 hour, 18 hour, and 48 hour intervals, respectively, at -80°C with DuPont Cronex intensifying screens. P=pith; C=cob; BR=brace roots; ES=ear shank; LP=lower pith; MP=middle pith; UP=upper pith; S=seed; L=leaf; R=root; SH=sheath; and P(upper left)=total pith.

Fig. 26 is a Northern blot analysis, the two left lanes of which show the maize TrpA gene expression in the leaf (L) and pith (P) of Funk inbred lines 211D and 5N984. The five right lanes indicate the absence of expression in Funk 211D seed total RNA. S(1, 2,3, 4 and 5)= seed at 1, 2, 3, 4 and 5 weeks post pollenation. L=leaf; P=pith; S#=seed # weeks post pollenation.

Fig. 27 is a Southern blot analysis of genomic DNA Funk line 211D, probed with maize TrpA cDNA 8-2 (pCIB5600), wherein B denotes BamHI, E denotes EcoRI, EV denotes EcoRV, H denotes HINDIII, and S denotes SacI. 1X, 5X and 10X denote reconstructed gene copy equivalents.

Fig. 28A is a primer extension analysis which shows the transcriptional start of the maize TrpA subunit gene and sequencing ladder at a 1 hour exposure against film at -80C with Dupont Cronex intensifying screens. Lane +1 and +2 are 1X + 0.5X samples of primer extension reaction.

Fig. 28B is an analysis of RNase protection from +2 bp to +387 bp at annealing temperatures of 42°C, 48°C and 54°C, at a 16 hour exposure against film at -80°C with DuPont Cronex intensifying screens.

Fig. 29 is A map of the original Type II pollen-specific cDNA clone. The subcloning of the three EcoRI fragments into pBluescript vectors to create pCIB3168, pCIB3169 and II-.6 is illustrated.

Fig. 30 shows the DNA sequence of the maize pollen-specific calcium dependent protein kinase gene cDNA (SEQ ID NO:20), as contained in the 1.0 kb and 0.5 kb fragments of the original Type II cDNA clone. The EcoRI site that divides the 1.0 kb and 0.5 kb fragments is indicated. This cDNA is not full length, as the mRNA start site maps 490 bp upstream of the end of the cDNA clone. The translated protein is disclosed as SEQ ID NO:21.

Fig. 31 illustrates the tissue-specific expression of the pollen CDPK mRNA. RNA from the indicated maize 211D tissues was denatured, electrophoresed on an agarose gel, transferred to nitrocellulose, and probed with the pollen CDPK cDNA 0.5 kb fragment. The mRNA is detectable only in the pollen, where a strong signal is seen.

Fig. 32 is an amino acid sequence (sequence line 1, amino acids 13 to 307 of SEQ ID NO:22) comparison of the pollen CDPK derived protein sequence and the rat calmodulin-dependent protein kinase 2 protein sequence (sequence line 3; SEQ ID NO:23) disclosed in Tobimatsu et al., J. Biol. Chem. 263:16082-16086 (1988). The Align program of the DNAstar software package was used to evaluate the sequences. The homology to protein kinases occurs in the 5' two thirds of the gene, i.e. in the 1.0 kb fragment.

Fig. 33 is an amino acid sequence comparison of the pollen CDPK derived protein sequence (sequence line 1; amino acids 311 to 450 of SEQ ID NO:22) and the human calmodulin protein sequence (sequence line 3; SEQ ID NO:24) disclosed in Fischer et al., J. Biol. Chem. 263:17055-17062 (1988). The homology to calmodulin occurs in the 3' one third of the gene, i.e. in the 0.5 kb fragment.

Fig. 34 is an amino acid sequence comparison of the pollen CDPK derived protein sequence (sequence line 1; SEQ ID NO:22) and soybean CDPK (SEQ ID NO:25). The homology occurs over the entire gene.

Fig. 35 illustrates the sequence of the maize pollen-specific CDPK gene (SEQ ID NO:26). 1.4 kb of sequence prior to the mRNA start site is shown. The positions of the seven exons and six introns are depicted under the corresponding DNA sequence. The site of polyadenylation in the cDNA clone is indicated.

Fig. 36 is a map of pCIB4433.

Fig. 37 is a full-length, hybrid, maize-optimized DNA sequence (SEQ ID NO:27) encoding a heat stable cryIA(b) protein (SEQ ID NO:28).

Fig. 38 is a map of pCIB5515.

DESCRIPTION OF THE SEQUENCES:

SEQ ID NO:1 is the DNA sequence of a full-length native Bt cryIA(b) gene.

SEQ ID NO:2 is the DNA sequence of a full-length pure maize optimized synthetic Bt cryIA(b) gene.

SEQ ID NO:3 is the DNA sequence of an approximately 2 Kb truncated synthetic maize optimized Bt cryIA(b) gene.

SEQ ID NO:4 is the DNA sequence of a full-length synthetic maize optimized Bt cryIA(b) gene.

SEQ ID NO:5 is the DNA sequence of an approximately 2 Kb synthetic Bt gene according to Perlak et al.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are provided in order to provide clarity with respect to the terms as they are used in the specification and claims to describe the present invention.

Maize preferred codon: Preferred codon refers to the preference exhibited by a specific host cell in the usage of nucleotide codons to specify a given amino acid. The preferred codon for an amino acid for a particular host is the single codon which most frequently encodes that amino acid in that host. The maize preferred codon for a particular amino acid may be derived from known gene sequences from maize. For example, maize codon usage for 28 genes from maize plants are listed in Table 4 of Murray et al., Nucleic Acids Research, 17:477-498 (1989),

the disclosure of which is incorporated herein by reference. For instance, the maize preferred codon for alanine is GCC, since, according to pooled sequences of 26 maize genes in Murray et al., supra, that codon encodes alanine 36% of the time, compared to GCG (24%), GCA (13%), and GCT (27%). Table 4 of Murray et al. is reproduced below.

Codon Usage In Pooled Sequences Of Higher Plant Genes.

AmAcid	Codon	Soybean n = 29 No.	%	Maize n = 26 No.	%	CAB n = 17 No.	%	RuBP SSU n = 20 No.	%
Gly	GGG	90	16	95	16	42	8	16	9
Gly	GGA	189	33	78	13	167	32	95	51
Gly	GGT	193	33	129	21	196	37	32	17
Gly	GGC	102	18	302	50	118	23	43	23
Glu	GAG	310	51	368	81	178	71	139	74
Glu	GAA	301	49	84	19	73	29	49	26
Asp	GAT	244	62	87	24	53	29	39	33
Asp	GAC	150	38	277	76	128	71	79	67
Val	GTG	219	37	227	40	62	21	93	36
Val	GTA	77	13	36	6	24	8	7	3
Val	GTT	227	38	99	17	118	39	87	33
Val	GTC	75	12	209	37	96	32	73	28
Ala	GCG	42	8	211	24	26	5	16	5
Ala	GCA	170	30	115	13	61	12	42	14
Ala	GCT	208	37	237	27	225	45	110	38
Ala	GCC	139	25	324	36	192	38	125	43
Arg	AGG	88	22	109	26	21	15	17	12
Arg	AGA	119	30	28	7	33	24	31	21
Ser	AGT	117	18	29	5	15	5	21	8
Ser	AGC	129	20	150	28	84	27	56	22
Lys	AAG	278	58	367	90	186	85	176	85
Lys	AAA	204	42	43	10	34	15	30	15
Asn	AAT	168	40	56	19	52	30	35	26
Asn	AAC	248	60	246	81	119	70	102	74
Met	ATG	184	100	210	100	111	100	115	100
Ile	ATA	109	24	35	8	10	6	1	1
Ile	ATT	219	49	100	24	61	40	63	43
Ile	ATC	118	27	284	68	83	54	83	56
Thr	ACG	29	7	114	26	10	6	5	3
Thr	ACA	128	29	48	11	35	22	21	13
Thr	ACT	151	35	72	16	61	38	59	36
Thr	ACC	124	29	212	47	54	34	79	48
Trp	TGG	82	100	84	100	99	100	86	100
End	TGA	5	18	7	26	15	88	2	11
Cys	TGT	63	40	29	21	16	39	7	9
Cys	TGC	95	60	110	79	25	61	72	91
End	TAG	9	32	14	52	0	0	1	5
End	TAA	14	50	6	22	2	12	16	84
Tyr	TAT	135	49	38	14	23	19	17	10
Tyr	TAC	139	51	240	86	99	81	151	90
Leu	TTG	175	24	116	13	118	30	79	36
Leu	TTA	79	11	28	3	15	4	6	3
Phe	TTT	166	46	69	20	106	40	32	20
Phe	TTC	193	54	278	80	160	60	125	80
Ser	TCG	39	6	89	16	17	5	10	4
Ser	TCA	125	19	56	10	46	15	48	19
Ser	TCT	140	22	75	14	83	26	33	13
Ser	TCC	94	15	145	27	69	22	89	34

AmAcid	Codon	Soybean No.	Soybean %	Maize No.	Maize %	CAB No.	CAB %	RuBP SSU No.	RuBP SSU %
Arg	CGG	17	4	54	13	7	5	1	1
Arg	CGA	41	10	13	3	6	4	3	2
Arg	CGT	70	18	45	11	50	36	48	33
Arg	CGC	64	16	165	40	20	15	44	31
Gln	CAG	181	41	311	59	36	37	75	51
Gln	CAA	261	59	219	41	60	62	73	49
His	CAT	124	63	49	29	16	32	4	18
His	CAC	73	37	122	71	34	68	18	82
Leu	CTG	75	10	289	31	29	7	27	12
Leu	CTA	60	8	78	9	6	2	9	4
Leu	CTT	184	26	147	16	134	34	56	25
Leu	CTC	148	21	261	28	88	23	43	20
Pro	CCG	55	8	149	27	29	10	13	6
Pro	CCA	346	47	126	23	137	47	72	34
Pro	CCT	236	32	109	20	73	25	60	29
Pro	CCC	95	13	164	30	54	18	66	31

n = the number of DNA sequences in the sample. **No.** is the number occurrences of a given codon in the sample. **%** is the percent occurrence for each codon within a given amino acid in the sample. (See text of Murray et al., Nucleic Acids Research, 17:477-498 (1989) for a description of the samples).

Pure maize optimized sequence: An optimized gene or DNA sequence refers to a gene in which the nucleotide sequence of a native gene has been modified in order to utilize preferred codons for maize. For example, a synthetic maize optimized Bt cryIA(b) gene is one wherein the nucleotide sequence of the native Bt cryIA(b) gene has been modified such that the codons used are the maize preferred codons, as described above. A pure maize optimized gene is one in which the nucleotide sequence comprises 100 percent of the maize preferred codon sequences for a particular polypeptide. For example, the pure maize optimized Bt cryIA(b) gene is one in which the nucleotide sequence comprises 100 percent maize preferred codon sequences and encodes a polypeptide with the same amino acid sequence as that produced by the native Bt cryIA(b) gene. The pure nucleotide sequence of the optimized gene may be varied to permit manipulation of the gene, such as by altering a nucleotide to create or eliminate restriction sites. The pure nucleotide sequence of the optimized gene may also be varied to eliminate potentially deleterious processing sites, such as potential polyadenylation sites or intron recognition sites.

It is recognized that "partially maize optimized," sequences may also be utilized. By partially maize optimized, it is meant that the coding region of the gene is a chimeric (hybrid), being comprised of sequences derived from a native insecticidal gene and sequences which have

been optimized for expression in maize. A partially optimized gene expresses the insecticidal protein at a level sufficient to control insect pests, and such expression is at a higher level than achieved using native sequences only. Partially maize optimized sequences include those which contain at least about 5% optimized sequences.

Full-length Bt Genes: Refers to DNA sequences comprising the full nucleotide sequence necessary to encode the polypeptide produced by a native Bt gene. For example, the native Bt cryIA(b) gene is approximately 3.5 Kb in length and encodes a polypeptide which is approximately 1150 amino acids in length. A full-length synthetic cryIA(b) Bt gene would be at least approximately 3.5 Kb in length.

Truncated Bt Genes: Refers to DNA sequences comprising less than the full nucleotide sequence necessary to encode the polypeptide produced by a native Bt gene, but which encodes the active toxin portion of the polypeptide. For example, a truncated synthetic Bt gene of approximately 1.9 Kb encodes the active toxin portion of the polypeptide such that the protein product exhibits insecticidal activity.

Tissue-preferred promoter: The term "tissue- preferred promoter" is used to indicate that a given regulatory DNA sequence will promote a higher level of transcription of an associated structural gene or DNA coding sequence, or of expression of the product of the associated gene as indicated by any conventional RNA or protein assay, or that a given DNA sequence will demonstrate some differential effect; i.e., that the transcription of the associated DNA sequences or the expression of a gene product is greater in some tissue than in all other tissues of the plant.

"Tissue-specific promoter" is used to indicate that a given regulatory DNA sequence will promote transcription of an associated coding DNA sequence essentially entirely in one or more tissues of a plant, or in one type of tissue, e.g. green tissue, while essentially no transcription of that associated coding DNA sequence will occur in all other tissues or types of tissues of the plant.

The present invention provides DNA sequences optimized for expression in plants, especially in maize plants. In a preferred embodiment of the present invention, the DNA sequences encode the production of an insecticidal toxin, preferably a polypeptide sharing substantially the amino acid sequence of an insecticidal crystal protein toxin normally produced

by *Bacillus thuringiensis*. The synthetic gene may encode a truncated or full-length insecticidal protein. Especially preferred are synthetic DNA sequences which encode a polypeptide effective against insects of the order Lepidoptera and Coleoptera, and synthetic DNA sequences which encode a polypeptide having an amino acid sequence essentially the same as one of the crystal protein toxins of *Bacillus thuringiensis* variety kurstaki, HD-1.

The present invention provides synthetic DNA sequences effective to yield high expression of active insecticidal proteins in plants, preferably maize protoplasts, plant cells and plants. The synthetic DNA sequences of the present invention have been modified to resemble a maize gene in terms of codon usage and G+C content. As a result of these modifications, the synthetic DNA sequences of the present invention do not contain the potential processing sites which are present in the native gene. The resulting synthetic DNA sequences (synthetic Bt IP coding sequences) and plant transformation vectors containing this synthetic DNA sequence (synthetic Bt IP genes) result in surprisingly increased expression of the synthetic Bt IP gene, compared to the native Bt IP gene, in terms of insecticidal protein production in plants, particularly maize. The high level of expression results in maize cells and plants that exhibit resistance to lepidopteran insects, preferably European Corn Borer and *Diatrea saccharalis*, the Sugarcane Borer.

The synthetic DNA sequences of the present invention are designed to encode insecticidal proteins from *Bacillus thuringiensis*, but are optimized for expression in maize in terms of G+C content and codon usage. For example, the maize codon usage table described in Murray et al., supra, is used to reverse translate the amino acid sequence of the toxin produced by the *Bacillus thuringiensis* subsp. kurstaki HD-1 cryIA(b) gene, using only the most preferred maize codons. The reverse translated DNA sequence is referred to as the pure maize optimized sequence and is shown as Sequence 4. This sequence is subsequently modified to eliminate unwanted restriction endonuclease sites, and to create desired restriction endonuclease sites. These modifications are designed to facilitate cloning of the gene without appreciably altering the codon usage or the maize optimized sequence. During the cloning procedure, in order to facilitate cloning of the gene, other modifications are made in a region that appears especially susceptible to errors induced during cloning by the polymerase chain reaction (PCR). The final sequence of the maize optimized synthetic Bt IP gene is shown in Sequence 2. A comparison of

the maize optimized synthetic Bt IP gene with the native kurstaki cryIA(b) Bt gene is shown in Fig. 1.

In a preferred embodiment of the present invention, the protein produced by the synthetic DNA sequence is effective against insects of the order *Lepidoptera* or *Coleoptera*. In a more preferred embodiment, the polypeptide encoded by the synthetic DNA sequence consists essentially of the full-length or a truncated amino acid sequence of an insecticidal protein normally produced by *Bacillus thuringiensis* var. *kurstaki* HD-1. In a particular embodiment, the synthetic DNA sequence encodes a polypeptide consisting essentially of a truncated amino acid sequence of the Bt CryIA(b) protein.

The insecticidal proteins of the invention are expressed in a plant in an amount sufficient to control insect pests, i.e. insect controlling amounts. It is recognized that the amount of expression of insecticidal protein in a plant necessary to control insects may vary depending upon species of plant, type of insect, environmental factors and the like. Generally, the insect population will be kept below the economic threshold which varies from plant to plant. For example, to control European corn borer in maize, the economic threshold is .5 eggmass/plant which translates to about 10 larvae/plant.

The methods of the invention are useful for controlling a wide variety of insects including but not limited to rootworms, cutworms, armyworms, particularly fall and beet armyworms, wireworms, aphids, corn borers, particularly European corn borers, sugarcane borer, lesser corn stalk borer, Southwestern corn borer, etc.

In a preferred embodiment of the present invention, the synthetic coding DNA sequence optimized for expression in maize comprises a G+C percentage greater than that of the native cryIA(b) gene. It is preferred that the G+C percentage be at least about 50 percent, and more preferably at least about 60 percent. It is especially preferred that the G+C percent be about 64 percent.

In another preferred embodiment of the present invention, the synthetic coding DNA sequence optimized for expression in maize comprises a nucleotide sequence having at least about 90 percent homology with the "pure" maize optimized nucleotide sequence of the native *Bacillus thuringiensis* cryIA(b) protein, more preferably at least about 95 percent homology, and most preferably at least about 98 percent.

Other preferred embodiments of the present invention include synthetic DNA sequences having essentially the DNA sequence of SEQ ID NO:4, as well as mutants or variants thereof; transformation vectors comprising essentially the DNA sequence of SEQ ID NO:4; and isolated DNA sequences derived from the plasmids pCIB4406, pCIB4407, pCIB4413, pCIB4414, pCIB4416, pCIB4417, pCIB4418, pCIB4419, pCIB4420, pCIB4421, pCIB4423, pCIB4434, pCIB4429, pCIB4431, pCIB4433. Most preferred are isolated DNA sequences derived from the plasmids pCIB4418 and pCIB4420, pCIB4434, pCIB4429, pCIB4431, and pCIB4433.

In order to construct one of the maize optimized DNA sequences of the present invention, synthetic DNA oligonucleotides are made with an average length of about 80 nucleotides. These oligonucleotides are designed to hybridize to produce fragments comprising the various quarters of the truncated toxin gene. The oligonucleotides for a given quarter are hybridized and amplified using PCR. The quarters are then cloned and the cloned quarters are sequenced to find those containing the desired sequences. In one instance, the fourth quarter, the hybridized oligonucleotides are cloned directly without PCR amplification. Once all clones of four quarters are found which contain open reading frames, an intact gene encoding the active insecticidal protein is assembled. The assembled gene may then be tested for insecticidal activity against any insect of interest including the European Corn Borer (ECB) and the sugarcane borer. (Examples 5A and 5B, respectively). When a fully functional gene is obtained, it is again sequenced to confirm its primary structure. The fully functional gene is found to give 100% mortality when bioassayed against ECB. The fully functional gene is also modified for expression in maize.

The maize optimized gene is tested in a transient expression assay, e.g. a maize transient expression assay. The native Bt cryIA(b) coding sequence for the active insecticidal toxin is not expressed at a detectable level in a maize transient expression system. Thus, the level of expression of the synthesized gene can be determined. By the present methods, expression of a protein in a transformed plant can be increased at least about 100 fold to about 50,000 fold, more specifically at least about 1,000 fold to at least about 20,000 fold.

Increasing expression of an insecticial gene to an effective level does not require manipulation of a native gene along the entire sequence. Effective expression can be achieved by

manipulating only a portion of the sequences necessary to obtain increased expression. A full-length, maize optimized CryIA(b) gene may be prepared which contains a protein of the native CryIA(b) sequence. For example, Figure 7 illustrates a full-length, maize optimized CryIA(b) gene which is a synthetic-native hybrid. That is, about 2kb of the gene (nucleotides 1-1938 SEQ ID NO:8) is maize optimized, i.e. synthetic. The remainder, C-terminal nucleotides 647-1155 SEQ ID NO:8, are identical to the corresponding sequence native of the CryIA(b) gene. Construction of the illustrated gene is described in Example 6, below.

It is recognized that by using the methods described herein, a variety of synthetic/native hybrids may be constructed and tested for expression. The important aspect of hybrid construction is that the protein is produced in sufficient amounts to control insect pests. In this manner, critical regions of the gene may be identified and such regions synthesized using preferred codons. The synthetic sequences can be linked with native sequences as demonstrated in the Examples below. Generally, N-terminal portions or processing sites can be synthesized and substituted in the native coding sequence for enhanced expression in plants.

In another embodiment of the present invention, the maize optimized genes encoding cryIA(b) protein may be manipulated to render the encoded protein more heat stable or temperature stable compared to the native cryIA(b) protein. It has been shown that the cryIA(b) gene found in *Bacillus thuringiensis* kurstaki HD-1 contains a 26 amino acid deletion, when compared with the cryIA(a) and cryIA(c) proteins, in the -COOH half of the protein. This deletion leads to a temperature-sensitive cryIA(b) protein. See M. Geiser, EP 0 440 581, entitled "Temperaturstabiles *Bacillus thuringiensis*-Toxin". Repair of this deletion with the corresponding region from the cryIA(a) or cryIA(c) protein improves the temperature stability of the repaired protein. Constructs of the full-length modified cryIA(b) synthetic gene are designed to insert sequences coding for the missing amino acids at the appropriate place in the sequence without altering the reading frame and without changing the rest of the protein sequence. The full-length synthetic version of the gene is assembled by synthesizing a series of double-stranded DNA cassettes, each approximately 300 bp in size, using standard techniques of DNA synthesis and enzymatic reactions. The repaired gene is said to encode a "heat stable" or "temperature-stable" cryIA(b) protein, since it retains more biological activity than its native counterpart when exposed to high temperatures. Specific sequences of maize optimized, heat

stable cryIA(b) genes encoding temperature stable proteins are set forth in Figs. 9 (SEQ ID NO:10), 11 (SEQ ID NO:12), 13 (SEQ ID NO:14), and 15 (SEQ ID NO:16), and are also described in Example 8A, below.

The present invention encompasses maize optimized coding sequences encoding other polypeptides, including those of other *Bacillus thuringiensis* insecticidal polypeptides or insecticidal proteins from other sources. For example, cryIB genes can be maize optimized, and then stably introduced into plants, particularly maize. The sequence of a maize optimized cryIB gene constructed in accordance with the present invention is set forth in Fig. 6 (SEQ ID NO:6).

Optimizing a Bt IP gene for expression in maize using the maize preferred codon usage according to the present invention results in a significant increase in the expression of the insecticidal gene. It is anticipated that other genes can be synthesized using plant codon preferences to improve their expression in maize or other plants. Use of maize codon preference is a likely method of optimizing and maximizing expression of foreign genes in maize. Such genes include genes used as selectable or scoreable markers in maize transformation, genes which confer herbicide resistance, genes which confer disease resistance, and other genes which confer insect resistance.

The synthetic cryIA(b) gene is also inserted into *Agrobacterium* vectors which are useful for transformation of a large variety of dicotyledenous plant species. (Example 44). Plants stably transformed with the synthetic cryIA(b) *Agrobacterium* vectors exhibit insecticidal activity. .

The native Bt cryIA(b) gene is quite A+T rich. The G+C content of the full-length native Bt cryIA(b) gene is approximately 39%. The G+C content of a truncated native Bt cryIA(b) gene of about 2 Kb in length is approximately 37%. In general, maize coding regions tend to be predominantly G+C rich. The modifications made to the Bt cryIA(b) gene result in a synthetic IP coding region which has greater than 50% G+C content, and has about 65% homology at the DNA level with the native cryIA(b) gene. The protein encoded by this synthetic CryIA(b) gene is 100% homologous with the native protein, and thus retains full function in terms of insect activity. The truncated synthetic CryIA(b) IP gene is about 2 Kb in length and the gene encodes the active toxin region of the native Bt *kurstaki* CryIA(b) insecticidal protein. The length of the protein encoded by the truncated synthetic CryIA(b) gene is 648 amino acids.

The synthetic genes of the present invention are useful for enhanced expression in transgenic plants, most preferably in transformed maize. The transgenic plants of the present invention may be used to express the insecticidal CryIA(b) protein at a high level, resulting in resistance to insect pests, preferably coleopteran or lepidopteran insects, and most preferably European Corn Borer (ECB) and Sugarcane Borer.

In the present invention, the DNA coding sequence of the synthetic maize optimized gene may be under the control of regulatory elements such as promoters which direct expression of the coding sequence. Such regulatory elements, for example, include monocot or maize and other monocot functional promoters to provide expression of the gene in various parts of the maize plant. The regulatory element may be constitutive. That is, it may promote continuous and stable expression of the gene. Such promoters include but are not limited to the CaMV 35S promoter; the CaMV 19S promoter; *A. tumefaciens* promoters such as octopine synthase promoters, mannopine synthase promoters, nopaline synthase promoters, or other opine synthase promoters; ubiquitin promoters, actin promoters, histone promoters and tubulin promoters. The regulatory element may be a tissue-preferential promoter, that is, it may promote higher expression in some tissues of a plant than in others. Preferably, the tissue-preferential promoter may direct higher expression of the synthetic gene in leaves, stems, roots and/or pollen than in seed. The regulatory element may also be inducible, such as by heat stress, water stress, insect feeding or chemical induction, or may be developmentally regulated. Numerous promoters whose expression are known to vary in a tissue specific manner are known in the art. One such example is the maize phosphoenol pyruvate carboxylase (PEPC), which is green tissue-specific. See, for example, Hudspeth, R.L. and Grula, J.W., Plant Molecular Biology 12:579-589, 1989). Other green tissue-specific promoters include chlorophyll a/b binding protein promoters and RubisCO small subunit promoters.

The present invention also provides isolated and purified pith-preferred promoters. Preferred pith-preferred promoters are isolated from graminaceous monocots such as sugarcane, rice, wheat, sorghum, barley, rye and maize; more preferred are those isolated from maize plants.

In a preferred embodiment, the pith-preferred promoter is isolated from a plant TrpA gene; in a most preferred embodiment, it is isolated from a maize TrpA gene. That is, the promoter in its native state is operatively associated with a maize tryptophan synthase-alpha

subunit gene (hereinafter "TrpA"). The encoded protein has a molecular mass of about 38kD. Together with another alpha subnit and two beta subunits, TrpA forms a multimeric enzyme, tryptophan synthase. Each subunit can operate separately, but they function more efficiently together. TrpA catalyzes the conversion of indole glycerol phosphate to indole. Neither the maize TrpA gene nor the encoded protein had been isolated from any plant before Applicants' invention. The *Arabidopsis thaliana* tryptophan synthase beta subunit gene has been cloned as described Wright et al., The Plant Cell, 4:711-719 (1992). The instant maize TrpA gene has no homology to the beta subunit encoding gene. The present invention also provides purified pollen-specific promoters obtainable from a plant calcium-dependent protein kinase (CDPK) gene. That is, in its native state, the promoter is operably linked to a plant CDPK gene. In a preferred embodiment, the promoter is isolated from a maize CDPK gene. By "pollen-specific," it is meant that the expression of an operatively associated structural gene of interest is substantially exclusively (i.e. essentially entirely) in the pollen of a plant, and is negligible in all other plant parts. By "CDPK," it is meant a plant protein kinase which has a high affinity for calcium, but not calmodulin, and requires calcium, but not calmodulin, for its catalytic activity.

To obtain tissue-preferred or tissue specific promoters, genes encoding tissue specific messenger RNA (mRNA) can be obtained by differential screening of a cDNA library. For example, a pith-preferred cDNA can be obtained by subjecting a pith cDNA library to differential screening using cDNA probes obtained from pith and seed mRNA. See, *Molecular Cloning, A Laboratory Manual,* Sambrook et al. eds. Cold Spring Harbor Press: New York (1989).

Alternately, tissue specific promoters may be obtained by obtaining tissue specific proteins, sequencing the N-terminus, synthesizing oligonucleotide probes and using the probes to screen a cDNA library. Such procedures are exemplified in the Experimental section for the isolation of a pollen specific promoter.

The scope of the present invention in regard to the pith-preferred and pollen-specific promoters encompasses functionally active fragments of a full-length promoter that also are able to direct pith-preferred or pollen-specific transcription, respectively, of associated structural genes. Functionally active fragments of a promoter DNA sequence may be derived from a promoter DNA sequence, by several art-recognized procedures, such as, for example, by cleaving the promoter DNA sequence using restriction enzymes, synthesizing in accordance with the

sequence of the promoter DNA sequence, or may be obtained through the use of PCR technology. See, e.g. Mullis et al., Meth. Enzymol. 155:335-350 (1987); Erlich (ed.), PCR Technology, Stockton Press (New York 1989).

Further included within the scope of the instant invention are pith-preferred and pollen-specific promoters "equivalent" to the full-length promoters. That is, different nucleotides, or groups of nucleotides may be modified, added or deleted in a manner that does not abolish promoter activity in accordance with known procedures.

A pith-preferred promoter obtained from a maize TrpA gene is shown in Fig. 24 (SEQ ID NO:18). Those skilled in the art, with this sequence information in hand, will recognize that pith-preferred promoters included within the scope of the present invention can be obtained from other plants by probing pith libraries from these plants with probes derived from the maize TrpA structural gene. Probes designed from sequences that are highly conserved among TrpA subunit genes of various species, as discussed generally in Example 17, are preferred. Other pollen-specific promoters, which in their native state are linked to plant CDPK genes other than maize, can be isolated in similar fashion using probes derived from the conserved regions of the maize CDPK gene to probe pollen libraries.

In another embodiment of the present invention, the pith-preferred or pollen-specific promoter is operably linked to a DNA sequence, i.e. structural gene, encoding a protein of interest, to form a recombinant DNA molecule or chimeric gene. The phrase "operably linked to" has an art-recognized meaning; it may be used interchangeably with "operatively associated with,""linked to," or "fused to".

The structural gene may be homologous or heterologous with respect to origin of the promoter and/or a target plant into which it is transformed. Regardless of relative origin, the associated DNA sequence will be expressed in the transformed plant in accordance with the expression properties of the promoter to which it is linked. Thus, the choice of associated DNA sequence should flow from a desire to have the sequence expressed in this fashion. Examples of heterologous DNA sequences include those which encode insecticidal proteins, e.g. proteins or polypeptides toxic or inhibitory to insects or other plant parasitic arthropods, or plant pathogens such as fungi, bacteria and nematodes. These heterologous DNA sequences encode proteins such as magainins, Zasloff, PNAS USA, 84:5449-5453 (1987); cecropins, Hultmark et al., Eur. J.

Biochem. 127:207-217 (1982); attacins, Hultmark et al., EMBO J. 2:571-576 (1983); melittin, gramicidin S, Katsu et al., Biochem. Biophys. Acta, 939:57-63 (1988); sodium channel proteins and synthetic fragments, Oiki et al. PNAS USA, 85:2395-2397 (1988); the alpha toxin of *Staphylococcus aureus*m Tobkes et al., Biochem., 24:1915-1920 (1985); apolipoproteins and fragments thereof, Knott et al., Science 230:37 (1985); Nakagawa et al., J. Am. Chem. Soc., 107:7087 (1985); alamethicin and a variety of synthetic amphipathic peptides, Kaiser et al., Ann. Rev. Biophys. Biophys. Chem. 16:561-581 (1987); lectins, Lis et al., Ann. Rev. Biochem., 55:35-68 (1986); protease and amylase inhibitors; and insecticidal proteins from *Bacillus thuringiensis*, particularly the delta-endotoxins from *B. thuringiensis*; and from other bacteria or fungi.

In a preferred embodiment of the invention, a pith-preferred promoter obtained from a maize TrpA sübunit gene or pollen-specific promoter obtained from a maize CDPK gene is operably linked to a heterologous DNA sequence encoding a *Bacillus thuringiensis* ("B.t.") insecticidal protein. These proteins and the corresponding structural genes are well known in the art. See, Hofte and Whiteley, Microbiol. Reviews, 53:242-255 (1989).

While it is recognized that any promoter capable of directing expression can be utilized, it may be preferable to use heterologous promoters rather than the native promoter of the protein of interest. In this manner, chimeric nucleotide sequences can be constructed which can be determined based on the plant to be transformed as well as the insect pest. For example, to control insect pests in maize, a monocot or maize promoter can be operably linked to a Bt protein. The maize promoter can be selected from tissue-preferred and tissue- specific promoters such as pith-preferred and pollen-specific promoters, respectively as disclosed herein.

In some instances, it may be preferred to transform the plant cell with more than one chimeric gene construct. Thus, for example, a single plant could be transformed with a pith-preferred promoter operably linked to a Bt protein as well as a pollen-specific promoter operably linked to a Bt protein. The transformed plants would express Bt proteins in the plant pith and pollen and to a lesser extent the roots, outer sheath and brace roots.

For various other reasons, particularly management of potential insect resistance developing to plant expressed insecticidal proteins, it is beneficial to express more than one insecticidal protein (IP) in the same plant. One could express two different genes (such as two

different *Bacillus thuringiensis* derived delta-endotoxins which bind different receptors in the target insect's midgut) in the same tissues, or one can selectively express the two toxins in different tissues of the same plant using tissue specific promoters. Expressing two Bt genes (or any two insecticidal genes) in the same plant using three different tissue specific promoters presents a problem for production of a plant expressing the desired phenotype. Three different promoters driving two different genes yields six different insecticidal genes that need to be introduced into the plant at the same time. Also needed for the transformation is a selectable marker to aid in identification of transformed plants. This means introducing seven different genes into the plant at the same time. It is most desired that all genes, especially the insecticidal genes, integrate into the plant genome at the same locus so they will behave as a single gene trait and not as a multiple gene trait that will be harder to track during breeding of commercial hybrids. The total number of genes can be reduced by using differential tissue specific expression of the different insecticidal proteins.

For example, by fusing cryIA(b) with the pollen and PEP carboxylase promoters, one would obtain expression of this gene in green tissues and pollen. Fusing a pith-preferred promoter with the cryIB delta endotoxin from *Bacillus thuringiensis* would produce expression of this insecticidal protein most abundantly in the pith of a transformed plant, but not in seed tissues. Transformation of a plant with three genes, PEP carboxylase/cryIA(b), pollen/cryIA(b), and pith/cryIB produces a plant expressing two different Bt insecticidal endotoxins in different tissues of the same plant. CryIA(b) would be expressed in the "outside" tissues of a plant (particularly maize), that is, in those tissues which European corn borer feeds on first after hatching. Should ECB prove resistant to cryIA(b) and be able to burrow into the stalk of the plant after feeding on leaf tissue and/or pollen, it would then encounter the cryIB delta-endotoxin and be exposed to a second insecticidal component. In this manner, one can differentially express two different insecticidal components in the same plant and decrease the total number of genes necessary to introduce as a single genetic unit while at the same time providing protection against development of resistance to a single insecticidal component.

Likewise, a plant may be transformed with constructs encoding more than one type of insecticidal protein to control various insects. Thus, a number of variations may be constructed by one of skill in the art.

The recombinant DNA molecules of the invention may be prepared by manipulating the various elements to place them in proper orientation. Thus, adapters or linkers may be employed to join the DNA fragments. Other manipulations may be performed to provide for convenient restriction sites, removal of restriction sites or superfluous DNA. These manipulations can be performed by art-recognized methods. See, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, second edition, 1989. For example, methods such as restriction, chewing back or filling in overhangs to provide blunt ends, ligation of linkers, complementary ends of the DNA fragments can be provided for joining and ligation. See, Sambrook et al., supra.

Other functional DNA sequences may be included in the recombinant DNA molecule, depending upon the way in which the molecule is to be incorporated into the target plant genome. For instance, in the case of *Agrobacterium*-mediated transformation, if Ti- or the Ri- plasmid is used to transform the plant cells, the right and left borders of the T-DNA of the Ti- and Ri-plasmid will be joined as flanking regions to the expression cassette. *Agrobacterium tumefaciens*-mediated transformation of plants has been described in Horsch et al., Science, 225:1229 (1985); Marton, Cell Culture Somatic Cell Genetics of Plants, 1:514-521 (1984); Hoekema, In: The Binary Plant Vector System Offset-Drukkerij Kanters B.V., Alblasserdam, 1985, Chapter V Fraley, et al., Crit. Rev. Plant Sci., 4:1-46; and An et al., EMBO J., 4:277-284 (1985).

The recombinant DNA molecules of the invention also can include a marker gene to facilitate selection in recombinant plant cells. Examples of markers include resistance to a biocide such as an antibiotic, e.g. kanamycin, hygromycin, chloramphenicol, paramomycin, methotrexate and bleomycin, or a herbicide such as imidazolones, sulfonylureas, glyphosate, phosphinothricin, or bialaphos. Marker genes are well known in the art.

In another embodiment of the present invention, plants stably transformed with a recombinant DNA molecule or chimeric gene as described hereinabove are provided. The resultant transgenic plant contains the transformed gene stably incorporated into its genome, and will express the structural gene operably associated to the promoter in the respective fashion.

Transgenic plants encompassed by the instant invention include both monocots and dicots. Representative examples include maize, tobacco, tomato, cotton, rape seed, soybean,

wheat, rice, alfalfa, potato and sunflower. Preferred plants include maize, particularly inbred maize plants.

All transformed plants encompassed by the instant invention may be prepared by several methods known in the art. *A. tumefaciens*-mediated transformation has been disclosed above. Other methods include direct gene transfer into protoplasts, Paszkowski et al., EMBO J., 12:2717 (1984); Loerz et al., Mol. Gen. & Genet., 1199:178 (1985); Fromm et al., Nature 319:719 (1986); microprojectile bombardment, Klein et al., Bio/Technology, 6:559-563 (1988); injection into protoplasts, cultured cells and tissues, Reich et al., Bio/Technology, 4:1001-1004 (1986); or injection into meristematic tissues or seedlings and plants as described by De La Pena et al., Nature, 325:274-276 (1987); Graves et al., Plant Mol. Biol., 7:43-50 (1986); Hooykaas-Van Slogteren et al., Nature, 311:763-764 (1984); Grimsley et al., Bio/Technology, 6:185 (1988); and Grimsley et al., Nature, 325:177 (1988); and electroporation, WO92/09696.

The expression pattern of a structural gene operatively associated with an instant tissue-preferred or tissue-specific promoter in a transformed plant containing the same is critical in the case where the structural gene encodes an insecticidal protein. For example, the instantly disclosed pith-preferred expression pattern will allow the transgenic plant to tolerate and withstand pathogens and herbivores that attack primarily the pith, but also the brace roots, outer sheath and leaves of the plant since the protein will be expressed to a lesser extent but still in an insect controlling amount in these plant parts, but yet in the case of both types of promoters, will leave the seed of the plant unaffected.

EXAMPLES

The following examples further describe the materials and methods used in carrying out the invention. They are offered by way of illustration, and not by way of limitation.

EXAMPLE 1: General Methods

DNA manipulations were done using procedures that are standard in the art. These procedures can often be modified and/or substituted without substantively changing the result. Except where other references are identified, most of these procedures are described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, second edition, 1989.

Synthesis of DNA oligomers:

DNA oligomers which are from about twenty to about ninety, preferably from about sixty to about eighty nucleotides in length, are synthesized using an Applied Biosystems model 380B DNA synthesizer and standard procedures. The oligomers are made using the updated SSCAF3 cycle on a 0.2 μmole, wide pore, small scale ABI column. The end procedure is run trityl off and the oligomer is cleaved from the column using the 380B's automatic cleavage cycle. The oligomers are then deblocked in excess ammonium hydroxide (NH_4OH) at 55°C for 8-12 hours. The oligomers are then dried in an evaporator using nitrogen gas. After completion, the oligomers are resuspended in 0.25 - 0.5 ml of deionized water.

Purification of synthetic oligomers:

An aliquot of each oligomer is mixed with an equal volume of blue dye\formamide mix with the final solution containing 0.05% bromophenol blue, 0.05% xylene cyanol FF, and 25 % formamide. This mixture is heated at 95°C for 10 minutes to denature the oligomers. Samples are then applied to a 12 % polyacrylamide-urea gel containing 7 M urea (Sambrook et al.). After electrophoresis at 300-400 volts for 3-4 hours using a Vertical Slab Gel Unit (Hoefer Scientific Instruments, San Francisco, CA), UV shadowing is used to locate the correct sized fragment in the gel which was then excised using a razor blade. The purified gel fragment is minced and incubated in 0.4 M LiCl, 1 mM EDTA (pH 8) buffer overnight at 37°C.

Either of two methods is used to separate the oligomers from the polyacrylamide gel remnants: Gene\X 25 μM porous polyethylene filter units or Millipore's ultrafree-MC 0.45 μM filter units. The purified oligomers are ethanol precipitated, recovered by centrifuging in a microfuge for 20 min at 4°C, and finally resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0). Concentrations are adjusted to 50 ng\|H 25l based on absorption readings at 260 nm.

Kinasing oligomers for size determinations:

To check the size of some of the oligomers on a sequencing gel, kinase labeling reactions are carried out using purified synthetic oligomers of each representative size: 40mers, 60mers, 70mers, 80mers, and 90mers. In each 20 μl kinasing reaction, one pmole of purified oligomer is used in a buffer of 7.0 mM Tris pH 7.5, 10 mM KCl, 1 mM MgCl2), 0.5 mM DTT, 50 μg/ml BSA, 3000 μCi (3 pmoles) of 32P-gammaATP, and 8 units of T4 polynucleotide kinase. The kinase reaction is incubated for 1 hour at 37°C, followed by a phenol\chloroform extraction and three ethanol precipitations with glycogen as carrier (Tracy, Prep. Biochem. 11:251-268 (1981).

Two gel loadings (one containing 1000 cpm, the other containing 2000 cpm) of each reaction are prepared with 25% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. The kinased oligomers are boiled for 5 minutes before loading on a 6 % polyacrylamide, 7 M urea sequencing gel (BRL Gel Mix TM6, BRL, Gaithersburg, MD). A sequencing reaction of plasmid pUC18 is run on the same gel to provide size markers. After electrophoresis, the gel is dried and exposed to diagnostic X-ray film (Kodak, X-OMAT AR). The resulting autoradiograph shows all purified oligomers tested to be of the correct size. Oligomers which had not been sized directly on the sequencing gel are run on a 6 % polyacrylamide, 7 M urea gel (BRL Gel Mix TM6), using the sized oligomers as size markers. All oligomers are denatured first with 25 % formamide at 100°C for 5 minutes before loading on the gel. Ethidium bromide staining of the polyacrylamide gel allows all the oligomers to be visualized for size determination.

Hybridizing oligomers for direct cloning:

Oligomers to be hybridized are pooled together (from 1 μg to 20 μg total DNA) and kinased at 37°C for 1 hour in 1X Promega ligation buffer containing 30 mM Tris-HCl pH 7.8, 10 mM MgCl2, 10 mM DTT, and 1 mM dATP. One to 20 units of T4 polynucleotide kinase is used in the reaction, depending on the amount of total DNA present. The kinasing reactions are stopped by placing the reaction in a boiling water bath for five minutes. Oligomers to form the 5' termini of the hybridized molecules are not kinased but are added to the kinased oligomers along with additional hybridization buffer after heating. The pooled oligomers are in a volume of 50-100 ul with added hybridization buffer used to adjust the final salt conditions to 100 mM NaCl, 120 mM Tris pH 7.5, and 10 mM MgCl2. The kinased and non-kinased oligomers are pooled together and heated in a boiling water bath for five minutes and allowed to slowly cool to room temperature over a period of about four hours. The hybridized oligomers are then phenol\chloroform extracted, ethanol precipitated, and resuspended in 17 μl of TE (10 mM Tris, 1 mM EDTA, pH 8.0). Using this 17 μl, a ligation reaction with a final volume of 20 μl is assembled (final conditions = 30 mM Tris-HCl pH 7.8, 10 mM MgCl2, 10 mM DTT, 1 mM ATP, and 3 units of T4 DNA ligase (Promega, Madison WI). The ligation is allowed to incubate for about 2 hours at room temperature. The hybridized\ligated fragments are generally purified on 2% Nusieve gels before and\or after cutting with restriction enzymes prior to cloning into vectors. A 20 μl volume ligation reaction is assembled using 100 ng to 500 ng of each fragment with approximate equimolar amounts of DNA in 30 mM Tris-HCl pH 7.8, 10 mM MgCl2, 10 mM DTT, 1 mM ATP, and 3 units of T4 DNA ligase (Promega, Madison, WI). Ligations are incubated at room temperature for 2 hours. After ligation, DNA is transformed into frozen competent *E. coli* cells using standard procedures (Sambrook et al.) and transformants are selected on LB-agar (Sambrook et al.) containing 100 μg/ml ampicillin (see below).

PCR Reactions for Screening clones in *E. coli*:

E. coli colonies which contain the correct DNA insert are identified using PCR (see generally, Sandhu et al., BioTechniques 7:689-690 (1989)). Using a toothpick, colonies are scraped from an overnight plate and added to a 20 μl to 45 μl PCR reaction mix containing about 50 pmoles of each hybridizing primer (see example using primers MK23A28 and MK25A28 to

select orientation of SacII fragment in pHYB2#6), 200 μm to 400 mM of each dNTP, and 1X reaction buffer (Perkin Elmer Cetus, Norwalk, CT). After boiling the *E. coli*\PCR mix in a boiling water bath for 10 minutes, 5 μl of Taq polymerase (0.5 units)(Perkin Elmer Cetus, Norwalk, Conn.) in 1X reaction buffer is added. The PCR reaction parameters are generally set with a denaturing step of 94°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 45 seconds for 30 to 36 cycles. PCR reaction products are run on agarose or Nusieve agarose (FMC) gels to detect the correct fragment size amplified.

Ligations:

Restriction enzyme digested fragments are either purified in 1% LGT (low gelling temperature agarose, FMC), 2% Nusieve (FMC), or 0.75% agarose using techniques standard in the art. DNA bands are visualized with ethidium bromide and bands are recovered from gels by excision with a razor blade. Fragments isolated from LGT are ligated directly in the LGT. Ten microliters of each recovered DNA fragment is used to assemble the ligation reactions, producing final ligation reaction volumes of about 23 μl. After excision with a razor blade, the recovered gel bands containing the desired DNA fragments are melted and brought to 1X ligase buffer and 3 units of T4 DNA ligase (Promega) are added as described above. Fragments isolated from either regular agarose or Nusieve agarose are purified from the agarose using ultrafree-MC 0.45 μM filter units (Millipore) and the fragments are ligated as described above. Ligation reactions are incubated at room temperature for two hours before transforming into frozen competent *E. coli* cells using standard procedures (Sambrook et al.).

Transformations:

Frozen competent *E. coli* cells of the strain DH5alpha or HB101 are prepared and transformed using standard procedures (Sambrook et al.). *E. Coli* "SURE" competent cells are obtained from Stratagene (La Jolla, CA). For ligations carried out in LGT agarose, after ligation reactions are complete, 50 mM CaCl2 is added to a final volume of about 150 μl and the solution heated at approximately 65°C for about 10 minutes to completely melt the agarose. The solution is then mixed and chilled on ice for about 10 minutes before the addition of about 200 μl of competent cells which had been thawed on ice. This mixture is allowed to incubate for 30

minutes on ice. The mixture is next heat shocked at 42°C for 60 seconds before chilling on ice for two minutes. Next, 800 μl of SOC media (20% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, adjusted to pH 8 with 5 N NaOH, 20 mM MgCl2:MgSO4 mix, and 20 mM glucose; Sambrook et al.) is added and the cells are incubated at 37°C with shaking for about one hour before plating on selective media plates. Plates typically are L-agar (Sambrook et al.) containing 100 μg/ml ampicillin.

When ligations are carried out in a solution without agarose, typically 200 μl of frozen competent *E. coli* cells (strain DH5alpha (BRL, Gaithersburg, MD or Sure cells, Stratagene, La Jolla, CA) are thawed on ice and 5 μl of the ligation mixture added. The reaction is incubated on ice for about 45 to 60 minutes, the cells are then heat shocked at 42° for about 90 seconds. After recovery at room temperature for about 10 minutes, 800 μl of SOC medium is added and the cells are then incubated 1 hour at 37°C with shaking and plated as above.

When screening for inserts into the beta-galactosidase gene in some of the standard vectors used, 200 μl of the recovered transformation mixture is plated on LB-agar plates containing 0.008% X-gal, 80 μM IPTG, and 100 μg/ml ampicillin (Sambrook et al.). The plates are incubated at 37° overnight to allow selection and growth of transformants.

Miniscreening DNA:

Transformants from the selective media plates are grown and their plasmid structure is examined and confirmed using standard plasmid mini-screen procedures (Sambrook et al.). Typically, the "boiling" procedure is used to produce small amounts of plasmid DNA for analysis (Sambrook et al.). Alternatively, an ammonium acetate procedure is used in some cases. This procedure is a modification of that reported by Shing-yi Lee et al., Biotechniques 9:676-679 (1990).

1) Inoculate a single bacterial colony from the overnight selection plates into 5 ml (can be scaled down to 1 ml) of TB (Sambrook et al.) medium and grow in the presence of the appropriate antibiotic.

2) Incubate on a roller at 37°C overnight.

3) Collect 5 ml of bacterial cells in a plastic Oakridge tube and spin for 5 min. at 5000 rpm in a Sorvall SS-34 rotor at 4°C.

4) Remove the supernatant.

5) Resuspend the pellet in 1 ml of lysis buffer (50 mM glucose, 25 mM Tris-HCl(pH 8.0), 10 mM EDTA and 5 mg/ml lysozyme), vortex for 5 seconds, and incubate at room temperature for 5 min.

6) Add 2 ml of freshly prepared alkaline solution (0.2 N NaOH, 1% sodium dodecyl sulfate), tightly secure lid, mix by inverting 5 times and place tube in an ice-water bath for 5 min.

7) Add 1.5 ml of ice-cold 7.5 M ammonium acetate (pH 7.6) to the solution, mix by inverting the tube gently 5 times and place on an ice-water bath for 5 min.

8) Centrifuge mixture at 9000 rpm for 10 min. at room temperature.

9) Transfer clear supernatant to a 15 ml Corex tube and add 0.6 volumes of isopropanol (approx. 2.5 ml). Let sit at room temperature for 10 min.

10) Centrifuge the mixture at 9000 rpm for 10 min. at room temperature and discard the supernatant.

11) Resuspend the pellet in 300 ul of TE buffer. Add 6 ul of a stock of RNase A & T1 (made as a 200 ul solution by adding 180 ul of RNase A (3254 Units/mg protein, 5.6 mg protein/ml) and 20 ul of RNase T1(481 Units/ug protein, 1.2 mg protein/ml)). These stocks may be purchased from USB(US Biochemical). Transfer to a microcentrifuge tube and incubate at 37°C for 15 min.

12) Add 75 ul of distilled water and 100 ul of 7.5 M ammonium acetate and incubate in an ice-water bath for 10 min.

13) Centrifuge the mixture at 14,000 rpm for 10 min. in a Beckman microfuge at 4°C.

14) Precipitate by adding 2.5 volumes of 100% EtOH (approx. 1 ml) and incubate in an ice-water bath for 10 min.

15) Spin at 14,000 rpm for 10 min. in a microfuge.

16) Wash pellet with 70% ethanol (using 0.5 ml-1 ml). Dry the pellet and resuspend in 100 μl of 1X New England Biolabs restriction enzyme Buffer 4 (20 mM Tris-HCl(pH 7.9), 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT). Measure concentration and check purity by spectrophotometry at absorbances 260 and 280 nm.

For a more rapid determination as to whether or not a particular bacterial colony harbored a recombinant plasmid, a PCR miniscreen procedure is carried out using a modification

of the method described by (Sandhu, G.S. et al., 1989, BioTechniques, 7:689-690). Briefly, the following mixture is prepared:

100 μl primer mix above, 20 μM each primer,

100 μl dNTP mix (2.5 mM each)

100 μl 10X AmpliTaq buffer (Perkin-Elmer Cetus, 1X buffer = 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, and 0.01% gelatin)

700 μl deionized water.

20 μl of the above mixture is put into a a 0.5 ml polyproplyene PCR tube. A transformed bacterial colony is picked with a toothpick and resuspended in the mixture. The tube is put in a boiling water bath for 10 minutes and then cooled to room temperature before adding 5 μl of the mix described below:

265 μl deionized water

30 μl 10X Amplitaq buffer (Perkin-Elmer Cetus, 1X buffer = 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, and 0.01% gelatin)

7.5 μl Taq polymerase

The samples are overlaid with 50 μl of mineral oil and PCR is carried out for 30 cycles using the following parameters:

denature: 94° for 1 min

anneal: 55° for 1 min

extend: 72° for 45 seconds.

After PCR amplification, 1 μl of loading dye (30% glycerol, 0.25% Bromophenol blue, 0.25% xylene cyanol) is added to the whole reaction and 20 μl of the mixture is loaded on a 2% Nusieve, 1% agarose gel to see if there is a PCR product of the expected size.

This procedure is used as an initial screen. Minipreps are subsequently carried out to confirm the structure of the plasmid and its insert prior to sequencing.

EXAMPLE 2: Amplification And Assembly Of Each Quarter

Cloning fragments of the synthetic Bt cryIA(b) gene:

The synthetic gene was designed to be cloned in four pieces, each roughly one quarter of the gene. The oligomers for each quarter were pooled to either be assembled by PCR, hybridization, or a combination of hybridization followed by PCR amplification as described elsewhere. Synthetic quarters were pieced together with overlapping restriction sites Aat II, NcoI, and Apa I between the 1st and 2nd, 2nd and 3rd, and 3rd and 4th quarters respectively.

Each quarter of the gene (representing about 500 bp) was assembled by hybridizing the appropriate oligomers and amplifying the desired fragment using PCR primers specific for the ends of that quarter. Two different sets of PCR reactions employing two sets of slightly different primers were used. The PCR products of the two reactions were designed to be identical except that in the first reaction there was an additional AATT sequence at the 5' end of the coding region and in the second reaction there was an AGCT sequence at the 3' end of a given quarter. When the products of the two reactions for a particular quarter were mixed (after removing the polymerase, primers and incomplete products), denatured, and subsequently re-annealed, a certain ratio (theoretically 50%) of the annealed product should have non-homologous overhanging ends. These ends were designed to correspond to the "sticky ends" formed during restriction digestion with EcoRI at the 5' end and Hind III at the 3' end of the molecule. The resulting molecules were phosphorylated, ligated into an EcoRI/HindIII digested and phosphatased Bluescript vector, and transformed into frozen competent *E. coli* strain DH5alpha. After selection, the *E. coli* colonies containing the desired fragment are identified by restriction digest patterns of the DNA. Inserts representing parts of the synthetic gene are subsequently purified and sequenced using standard procedures. In all cases, clones from multiple PCR reactions are generated and sequenced. The quarters are then joined together using the unique restriction sites at the junctions to obtain the complete gene.

Cloned quarters are identified by mini-screen procedures and the gene fragment sequenced. It is found that errors are frequently introduced into the sequence, most probably during the PCR amplification steps. To correct such errors in clones that contain only a few such errors, hybridized oligomers are used. Hybridized fragments are digested at restriction enzyme recognition sites within the fragment and cloned to replace the mutated region in the synthetic

gene. Hybridized fragments range from 90 bp in length (e.g. the region that replaces the fragment between the Sac II sites in the 2nd quarter) to the about 350 bp 4th quarter fragment that replaces two PCR induced mutations in the 4th quarter of the gene.

Due to the high error rate of PCR, a plasmid is designed and constructed which allows the selection of a cloned gene fragment that contains an open reading frame. This plasmid is designed in such a manner that if an open reading frame is introduced into the cloning sites, the transformed bacteria could grow in the presence of kanamycin. The construction of this vector is described in detail below. This selection system greatly expedites the progress by allowing one to rapidly identify clones with open reading frames without having to sequence a large number of independent clones. The synthetic quarters are assembled in various plasmids, including BSSK (Stratagene; La Jolla, Ca), pUC18 (Sambrook et al.), and the Km-expression vector. Other suitable plasmids, including pUC based plasmids, are known in the art and may also be used. Complete sequencing of cloned fragments, western blot analysis of cloned gene products, and insect bioassays using European corn borer as the test insect verify that fully functional synthetic Bt cryIA(b) genes have been obtained.

Construction of the Km-expression vector to select open reading frames:

The Km-expression vector is designed to select for fragments of the synthetic gene which contain open-reading frames. PCR oligomers are designed which allow the fusion of the NPTII gene from Tn5 starting at nucleotide 13 (Reiss et al., EMBO J. 3:3317-3322 (1984)) with pUC18 and introduce useful restriction sites between the DNA segments. The polylinker region contains restriction sites to allow cloning various synthetic Bt IP fragments in-frame with the Km gene. The 88 bp 5' oligomer containing the polylinker region is purified on a 6% polyacrylamide gel as described above for the oligomer PAGE purification. A PCR reaction is assembled with a 1 Kb Bgl II\Sma I template fragment which contains the NPT II gene derived from Tn5. The PCR reaction mix contains 100 ng of template with 100 pmols of oligomers KE72A28 and KE74A28 (see sequences below), 200 nM dNTP, and 2.5 Units of Taq polymerase all in a 50 μl volume with an equal volume of mineral oil overlaid. Sequences of the primers are:

KE74A28

5'-GCAGATCTGG ATCCATGCAC GCCGTGAAGG GCCCTTCTAG AAGGCCTATC GATAAAGAGC TCCCCGGGGA TGGATTGCAC GCAGGTTC-3' (SEQ ID NO:29)

KE72A28

5'-GCGTTAACAT GTCGACTCAG AAGAACTCGT CAAGAAGGCG-3' (SEQ ID NO:30)

The PCR parameters used are: 94°C for 45 seconds (sec), 55°C for 45 sec, and 72°C for 55 sec with the extension at step 3 for 3 sec for 20 cycles. All PCR reactions are carried out in a Perkin-Elmer Cetus thermocycler. The amplified PCR product is 800 bp and contains the polylinker region with a translational start site followed by unique restriction sites fused in-frame with the Km gene from base #13 running through the translational terminator. pUC:KM74 is the Km-expression cassette that was assembled from the 800 bp Bgl II\Sal I polylinker/Km fragment cloned in the PUC18 vector. The lacZ promoter allows the Km gene to be expressed in *E. coli*. pUC:KM74 derivatives has to first be plated on LB-agar plates containing 100 μg/ml ampicillin to select transformants which can subsequently be screened on LB-agar plates containing 25 μg/ml kanamycin/IPTG. Synthetic Bt IP gene fragments are assembled from each quarter in the Km-cassette to verify cloning of open-reading-frame containing fragments pieces. The first ECB active synthetic Bt IP gene fragment, pBt:Km#6, is a Bt IP gene that shows Km resistance. This fragment is subsequently discovered to contain mutations in the 3rd and 4th quarter which are later repaired.

EXAMPLE 2A: Synthesis And Cloning Of The First Quarter Of The Synthetic Gene (base pairs 1 to 550)

The following procedures are followed in order to clone the first quarter of the synthetic DNA sequence encoding a synthetic Bt cryIA(b) gene. The same procedures are essentially followed for synthesis and cloning of the other quarters, except as noted for primers and restriction sites.

Template for Quarter 1: Mixture of equal amounts of purified oligomers U1-U7 and L1 to L7

PCR Primers:

Forward:

P1 (a): 5'-GTCGACAAGG ATCCAACAAT GG-3' (SEQ ID NO:31)

P1 (b): 5'-AATTGTCGAC AAGGATCCAA CAATGG-3' (SEQ ID NO:32)

Reverse:

P2 (a): 5'-ACACGCTGAC GTCGCGCAGC ACG-3' (SEQ ID NO:33)

P2 (b): 5'-AGCTACACGC TGACGTCGCG CAG-3' (SEQ ID NO:34)

Primer pair A1: P1(b) + P2(a)

Primer pair A2: P1(a) + P2(b)

The PCR reaction containing the oligomers comprising the first quarter of the synthetic maize-optimized Bt IP gene is set up as follows:

200 ng oligo mix (all oligos for the quarter mixed in equal amounts based on weight)

10 μl of primer mix (1:1 mix of each at 20 μM; primers are described above)

5 μl of 10X PCR buffer

PCR buffer used may be either

(a) 1X concentration = 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl, pH 8.0, 2 mM MgSO4, and 0.1% Triton X-100), or

(b) 1X concentration = 10mM Tris-HCl pH 8.3, 50 mM KCl 1.5mM $MgCl_2$, 0.01% wt/vol gelatin.

Components are mixed, heated in a boiling water bath for 5 minutes, and incubated at 65°C for 10 minutes.

Next, the following reagents are added:

8 μl of dNTPs mixture (final concentration in the reaction = 0.2 mM each)

5 units polymerase.

The final reaction volume is 50 microliters.

Oligomers are then incubated for 3 min at 72°C and then a PCR cycle is run. The PCR reaction is run in a Perkin Elmer thermocycler on a step cycle protocol as follows:

denaturation cycle : 94° for 1 minute

annealing cycle : 60° for 1 minute

extension cycle : 72° for 45 seconds (+ 3 sec per cycle)

number of cycles: 15

After the reaction is complete, 10 μl of the PCR reaction is loaded on a 2% Nusieve-GTG (FMC), 1% agarose analytical gel to monitor the reaction. The remaining 40 μl is used to clone the gene fragments as described below.

PCR Products

The termini of the double stranded PCR product corresponding to the various primer pairs are shown (only upper strand):

A1 AATTGTCGAC (SEQ ID NO:35) _____GCGTGT (554 bp) first qtr.

A2 GTCGAC_____GCGTGTAGCT (SEQ ID NO:36) (554 bp) first qtr.

Hybridization

40 μl of each of the PCR reactions described above is purified using a chromaspin 400 column (Clonetech, Palo Alto, CA) according to manufacturers directions. Five μg of carrier DNA was added to the reactions before loading on the column. (This is done for most of the cloning. However, in some reactions the PCR reactions are phenol:chloroform extracted using standard procedures (Sambrook et al.) to remove the Taq polymerase and the PCR generated DNA is recovered from the aqueous phase using a standard ethanol precipitation procedure.) The carrier DNA does not elute with the PCR generated fragments. The A1 and A2 reaction counterparts for each quarter are mixed, heated in a boiling water bath for 10 minutes and then incubated at 65°C overnight. The reactions are then removed from the 65° bath and ethanol

precipitated with 1 μl (20 μg) of nuclease free glycogen (Tracy, Prep. Biochem. 11:251-268 (1981) as carrier. The pellet is resuspended in 40 μl of deionized water.

Phosphorylation reaction

The phosphorylation reaction is carried out as follows:

40 μl DNA

2.5 μl 20 mM ATP

0.5 μl 10X BSA/DTT (1X = 5 mM DTT, 0.5 mg/ml BSA)

1.0 μl 10X polynucleotide kinase buffer (1X = 70 mM Tris.HCl, pH 7.6, 0.1 M KCl, 10 mM MgCl2)

2.0 μl polynucleotide kinase (New England Biolabs, 20 units).

Incubation is for 2 hours at 37°C.

The reaction is then extracted one time with a 1:1 phenol:chloroform mixture, then once with chloroform and the aqueous phase ethanol precipitated using standard procedures. The pellet is resuspended in 10 μl of TE.

Restriction Digests

20 μg of Bluescript vector (BSSK+, Stratagene, La Jolla, CA)

10 μl 10 X restriction buffer (1X = 20 mM Tris-HCl pH 8.0, 10 mM MgCl2, 100 mM NaCl)

5 μl Eco RI (New England Biolabs) 100 units

5 μl Hind III (New England Biolabs) 100 units

Final reaction volume is 100 μl.

Incubation is for 3 hours at 37°.

When completed, the reaction is extracted with an equal volume of phenol saturated with TE (10 mM Tris.HCl pH 8.0 and 10 mM EDTA). After centrifugation, the aqueous phase was extracted with an equal volume of 1:1 mixture of (TE saturated) phenol:chloroform (the "chloroform" is mixed in a ratio of 24:1 chloroform:isoamyl alcohol), and finally the aqueous phase from this extraction is extracted with an equal volume of chloroform. The final aqueous phase is ethanol

precipitated (by adding 10 μl of 3 M sodium acetate and 250 μl of absolute ethanol, left at 4° for 10 min and centrifuged in a microfuge at maximum speed for 10 minutes. The pellet is rinsed in 70% ethanol and dried at room temperature for 5-10 minutes and resuspended in 100 μl of 10 mM Tris.HCl (pH 8.3).

Phosphatase reaction

Vector DNA is routinely treated with phosphatase to reduce the number of colonies obtained without an insert. Calf intestinal alkaline phosphatase is typically used (Sambrook et al.), but other phosphatase enzymes can also be used for this step.

Typical phosphatase reactions are set up as below:

90 μl of digested DNA described above

10 μl of 10X Calf intestinal alkaline phosphatase buffer (1X=50 mM Tris-HCl (pH 8.3), 10 mM MgCl2, 1 mM ZnCl2, 10 mM spermidine)

1 μl (1 unit) of calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim, Indianapolis, IN)

Incubation is at 37°C for 1 hour.

The DNA is then gel purified (on a 1% low gelling temperature (LGT) agarose gel) and the pellet resuspended in 50 μl TE. After electrophoresis, the appropriate band is excised from the gel using a razor blade, melted at 65° for 5 minutes and diluted 1:1 with TE. This solution is extracted twice with phenol, once with the above phenol:chloroform mixture, and once with chloroform. The final aqueous phase is ethanol precipitated and resuspended in TE buffer.

Ligation:

To ligate fragments of the synthetic gene into vectors, the following conditions are typically used.

5 μl of phosphorylated insert DNA

2 μl of phosphatased Eco RI/Hind III digested Bluescript vector heated at 65° for 5 minutes, then cooled

1 μl 10X ligase buffer (1X buffer=30 mM Tris.HCl (pH 7.8), 10 mM MgCl2, 10 mM DTT, 1 mM ATP)

1 μl BSA (1 mg/ml)

1 μl ligase (3 units, Promega, Madison, Wisc.)

Ligase reactions are typically incubated at 16° overnight or at room temperature for two hours.

Transformation:

Transformation of ligated DNA fragments into *E. coli* is performed using standard procedures (Sambrook et al.) as described above.

Identification of recombinants

White or light blue colonies resulting from overnight incubation of transformation plates are selected. Plasmids in the transformants are characterized using standard mini-screen procedures (Sambrook et al.) or as described above. One of the three procedures listed below are typically employed:

(1) boiling DNA miniprep method

(2) PCR miniscreen

(3) Ammonium acetate miniprep.

The restriction digest of recombinant plasmids believed to contain the first quarter is set up as follows:

(a) Bam HI/Aat II digest: 10 μl DNA + 10 μl 1X New England Biolabs restriction enzyme Buffer 4

0.5 μl Bam HI (10 units)

0.5 μl Aat II (5 units)

Incubation is for about 2 hours at 37°C.

Clones identified as having the desired restriction pattern are next digested with Pvu II and with Bgl II in separate reactions. Only clones with the desired restriction patterns with all three enzyme digestions are carried further for sequencing.

Sequencing of cloned gene fragments:

Sequencing is performed using a modification of Sanger's dideoxy chain termination method (Sambrook et al.) using double stranded DNA with the Sequenase 2 kit (United States Biochemical Corp., Cleveland, OH). In all, six first quarter clones are sequenced. Of the clones

sequenced, only two clones designated pQA1 and pQA5 are found to contain only one deletion each. These deletions are of one base pair each located at position 452 in pQA1 and position 297 in pQA5.

Plasmid pQA1 is used with pP1-8 (as described below) to obtain a first quarter with the expected sequence.

EXAMPLE 2B: Synthesis And Cloning Of The Second Quarter (base pairs 531 to 1050)

Template: oligomers U8-U14 and L8-L14

PCR Primers:

forward:

P3 (a): 5'-GCTGCGCGAC GTCAGCGTGT TCGG-3' (SEQ ID NO:37)

P3 (b): 5'-AATTGCTGCG CGACGTCAGC GTG-3' (SEQ ID NO:38)

Reverse:

P4 (a): 5'-GGCGTTGCCC ATGGTGCCGT ACAGG-3' (SEQ ID NO:39)

P4 (b): 5'-AGCTGGCGT TGCCCATGGT GCCG-3' (SEQ ID NO:40)

Primer pair B1: P3(b) + P4(a)

Primer pair B2: P3(a) + P4(b)

PCR Products

B1 AATTGCTGCG (SEQ ID NO:41) _____AACGCC (524 bp) second quarter

B2 GCTGCG_____AACGCCAGCT (SEQ ID NO:42) (524 bp)

Hybridization, PCR amplification, spin column size fractionation, and cloning of this gene fragment in Bluescript digested with Eco RI/Hind III are performed as described above for the first quarter (Example 2A). The PCR product for this quarter is about 529 bp in size representing the second quarter of the gene (nucleotides 531 to 1050). Transformation is into

frozen competent *E. coli* cells (DH5alpha) using standard procedures described above (Sambrook et al.)

Miniscreen of pQB clones:

Miniprep DNA is prepared as described above and digested with (a) Aat II/Nco I, (b) Pvu II and (c) with Bgl I to confirm the structure insert in the vector before sequencing.

Sequencing is performed as described above using the dideoxy method of Sanger (Sambrook et al.).

A total of thirteen clones for this quarter are sequenced. The second quarter consistently contains one or more deletions between position 884 and 887. In most cases the G at position 884 is deleted.

Plasmid pQB5 had only one deletion at position 884. This region lies between two Sac II sites (positions 859 and 949). Correction of this deletion is described in Example 3.

Clones of the first half (1-1050 bp).

A fragment for cloning the first half (quarters 1 and 2) of the synthetic Bt maize gene as a single DNA fragment is obtained by restriction digestion of the product of a PCR reaction comprising the first quarter and the second quarter. Restriction endonuclease Aat II is used to cut the DNA (following phenol extraction and ethanol precipitation) in a 20 μl reaction. 15 μl of each of the Aat II digested quarters is mixed and ligated (in a 50 μl volume by adding 5 μl of 10X ligase buffer, (1X=30 mM Tris-HCl pH 7.8, 10 mM MgCl2, 10 mM DTT, 1 mM ATP) 14 μl of deionized water and 1 μl of T4 DNA ligase, 3 units, Promega, Madison, WI) at room temperature for 2 hr. The result is an about 1 kb fragment as judged by electrophoresis on a 1% agarose gel run using standard conditions (Sambrook et al.) Ten μl of the ligation product is amplified by PCR using conditions described previously except that only 5 cycles were run.

Primer Pair: HA= P1(a) + P4(b)

Primer Pair: HB= P1(b) + P4(a)

The product of these reactions is cloned into Bluescript (Stratagene, La Jolla, CA) as described for the individual quarters. This procedure is only done once i.e., all insert DNA is obtained in a particular region from a single PCR reaction.

Thirty-six colonies are miniscreened with Sal I digests and Pvu II digests. All except 4 contain an insert of approximately 1 kb in size of which at least 20 contain the correct Pvu II digestion pattern. Eight of these clones are selected for sequence analysis. One of the clones, P1-8, has the desired sequence between the Eco NI site (396 bp) and the Dra III site (640 bp). This clone is used to obtain a plasmid with the desired sequence up to the Dra III site (640 bp) in the second quarter with pQA1 (first quarter with a deletion at position 452 bp described previously.)

EXAMPLE 2C: Cloning And Synthesis Of Third Quarter (base pairs 1021 to 1500)

Template: Oligos U15-U20 and L15-L21

PCR primers:

forward

P5 (a): 5'-TTCCCCCTGT ACGGCACCAT GGGCAACGCC GC-3' (SEQ ID NO:43)

P5 (b): 5'-AATTGTACGG CACCATGGGC AAC-3' (SEQ ID NO:44)

reverse

P6 (a): 5'-GAAGCCGGGG CCCTTCACCA CGCTGG-3' (SEQ ID NO:45)

P6 (b): 5'-AGCTGAAGCC GGGGCCCTTC ACC-3' (SEQ ID NO:46)

Primer pair C1: P5(b) + P6(a)

Primer pair C2: P5(a) + P6(b)

PCR Product:

C1 AATTGTACGG (SEQ ID NO:47) _____GGCTTC (475 bp) 3d qtr

C2 TTCCCCTGTACGG (SEQ ID NO:48) _____GGCTTCAGCT (SEQ ID NO:49) (484 bp) 3d qtr

PCR reactions, spin column recovery of the correct sized DNA fragment, and ligation into vectors are performed as described above (Example 2A) using a Bluescript vector cut with Eco RI and Hind III. The approximately 479 base pair PCR product represents the third quarter of the synthetic gene (NT 1021 - 1500).

Transformation into frozen competent *E. coli* strain DH5alpha cells, selection and identification of transformants, characterization of transformants by mini-screen procedures, and sequencing of the synthetic gene fragment in the vector are all as described above.

Mini screen of pQC clones:

The third quarter is miniscreened using standard procedures (Sambrook et al.). Miniprep DNA is cut with (a) Nco I/Apa I and (b) with Pvu II. Clones containing the correct restriction digest patterns are sequenced using standard procedures. A total of 22 clones of the third quarter are sequenced. Three major deletion "hotspots" in the third quarter are identified (a) at position 1083 (b) between position 1290-1397 and (c) between positions 1356-1362. In all clones except one, pQC8, there is also consistently an insertion of a C at position 1365. In addition to these mutations, the third quarter clones contain a large number of other apparently random deletions. The common factor to the three mutational "hotspots" in the third quarter and the one in the second quarter is that these regions are all flanked on either side by sequences that are about 80% C+G. Other regions containing 5 to 9 C-Gs in a row are not affected. The oligomers in U15, U16, U18, U19, L15, L16, L18 and L19 are redesigned to reduce the C+G content in these regions. Five clones each from PCR reaction using the modified oligomers are sequenced.

Plasmid pQCN103 has the correct sequence for the third quarter except for a change at position 1326. This change, which substitutes a G for a C, results in the substitution of one amino acid (leucine) for the original (phenylalanine).

EXAMPLE 2D: Synthesis And Cloning Of Fourth Quarter (base pairs 1480 to 1960)

The fourth quarter of the gene is obtained from a clone which is originally designed to comprise the third and fourth quarters of the gene. The "second half" of the synthetic gene is obtained from PCR reactions to fuse the third and fourth quarters. These reactions are run with

PCR primers P5(a) and P6(a) described above for the third quarter and primers P7(a) and P8(a) (described below). The reverse primer is modified to include a Sac I site and a termination codon. Separate reactions for each quarter are run for 30 cycles using the conditions described above. The two quarters are joined together by overlapping PCR and subsequently digested with restriction enzymes Nco I and Sac I. The resulting 953 bp fragment is cloned directionally into pCIB3054, which has been cut with Nco I/Sac I and treated with alkaline phosphatase.

pCIB3054 is constructed by inserting intron #9 of PEPcarboxylase (PEPC ivs #9) in the unique Hpa I site of pCIB246 (described in detail in Example 4) pCIB246 is cut with HpaI and phosphatased with CIP using standard procedures described in Example 2A. PEPC ivs #9 is obtained by PCR using pPEP-10 as the template. pPEP-10 is a genomic subclone containing the entire maize PEP carboxylase gene encoding the C4 photosynthetic enzyme, plus about 2.2 Kb of 5'-flanking and 1.8 Kb of 3'-flanking DNA. The 10Kb DNA is ligated in the HindIII site of pUC18. (Hudspeth et al., Plant Molecular Biology, 12: 576-589 (1989). The forward PCR primer used to obtain the PEPCivs#9 is GTACAAAAACCAGCAACTC (SEQ ID NO:50) and the reverse primer is CTGCACAAAGTGGAGTAGT (SEQ ID NO:51). The PCR product is a 108 bp fragment containing only the PEPcarboxylase intron #9 sequences. The PCR reaction is extracted with phenol and chloroform, ethanol precipitated phosphorylated with polynucleotide kinase and treated with T4 polymerase to fill in the 3' nontemplated base addition found in PCR products (Clark, J.M., Nucleic Acid Research, 16: 9677-9686 (1988)) using standard procedures. The kinased fragment is blunt-end cloned into the HpaI site of pCIB246, using standard procedures described earlier.

Amplification and Assembly of the Fourth Quarter

Template: U21-U26 and L22-L28

<u>PCR primers</u>

FORWARD

P7 (a): 5'-TGGTGAAGGG CCCCGGCTTC ACCGG-3' (SEQ ID NO:52)

REVERSE

P8 (a): 5'-ATCATCGATG AGCTCCTACA CCTGATCGAT GTGGTA-3' (SEQ ID NO:53)

PRIMER PAIR 4: P7(a) + P8(a)

PRIMER PAIR 3: P5(A) + P6(a)

Primer pair for overlapping PCR : P7(a) + P8(a)

PCR Product

fourth quarter: GGTGAA_____ATCAGGAGCTCATCGATGAT (SEQ ID NO:54)

(484 bp) third quarter: TTCCCCCTGTA (SEQ ID NO:55)----------TTCACCGG

(484 bp) second half: GGTGAA-------CATGATGAT (953 bp)

Four positive clones are identified by plasmid miniscreen and are subsequently sequenced using standard procedures.

Plasmid Bt.P2 #1 contains approximately the correct fourth quarter sequence except for two mutations. These mutations are at position 1523 (substituting an A for a G, resulting in an amino acid change which substitutes a His for an Arg) and at position 1634 (substituting a T for a C, resulting in an amino acid substitution of a Ser for a Thr).

Plasmid Bt.P2 #1 is used in the construction of pCIB4414 described below. (The mistakes are ultimately corrected by hybridizing all the oligos of the fourth quarter, digesting with Apa I/Bst E II and replacing that region in pCIB4414. Therefore, only sequences from position 1842-1960 remain of Bt.P2#1 in the final construct.)

EXAMPLE 3: Assembly And Repair Of The Final Synthetic Gene

The synthetic maize optimized Bt cryIA(b) gene is designed to be cloned in quarters. Using the PCR technique, however, results in mutations, which in most cases are deletions resulting in frameshift mutations. Plasmids containing individual quarters are therefore sequenced and the correct parts ligated together using standard procedures.

After obtaining first and second quarter clones with almost the desired sequence, plasmids pEB1Q#4 and pEB1Q#5 are constructed to obtain the desired sequence of the synthetic Bt gene up to the Dra III site at the base pair position 634 (this mutation destroys the Dra III site). The pEB1Q constructs are made by ligating a 3.9 Kb Eco NI\Bam HI fragment from pP1-8 with a 400 bp fragment from pQA1. pEB1Q#5 has the desired sequence up to the Dra III site, but pEB1Q#4 has a mutation at base pair position 378.

Plasmids p1H1M4 and p1H1M5 are constructed to repair the Dra III site in pEB1Q#4 and pEB1Q#5. Plasmids p1H1M#4 and #5 are made by ligating a 3.5 Kb Nco I\Aat II fragment from pEB1Q#4 and #5 respectively, with a 500 bp Nco I\Aat II fragment from pQB5. Plasmid p1H1M5 contains a mutation between the Sac II sites at position 884 in the second quarter of the synthetic Bt gene. Plasmid p1H1M4 contains the additional mutation as described in its precursor construct pEB1Q#4.

The Sac II site in the Bluescript vector region of p1H1M4 is deleted by cutting p1H1M4 with Not I and Sac I and converting these sites to blunt ends using T4 DNA polymerase under standard conditions before ligating this 3.9 Kb fragment to make p1H1M4^S. Deleting the Sac II site in the vector region allows the 90 bp Sac II fragment with the mutation at position 884 in the 2nd quarter of p1H1M4^S to be removed prior to replacement with a 90 bp Sac II fragment. Oligomers U\L 12 and 13 are kinased and hybridized (described above) before cutting with Sac II and isolating a 90 bp fragment on a 2% Nusieve gel. The Sac II fragment is ligated into the about 3.8 Kb Sac II cut p1H1M4^S vector which has been phosphatased with CIP. The repaired Sac II construct is called pHYB2#6. The orientation of the Sac II fragment in pHYB2#6 is detected by PCR screening as described earlier using the following primers:

MK23A28 = 5'-GGGGCTGCGGATGCTGCCCT-3' (SEQ ID NO:56)
MK25A28 = 5'-GAGCTGACCCTGACCGTGCT-3' (SEQ ID NO:57)
MK26A28 = 5'-CACCTGATGGACATCCTGAA-3' (SEQ ID NO:58)

Running the PCR reactions with 50 pmoles of primers MK23A28 and MK25A28 produces an approximate 180 bp fragment, indicating the inserted fragment bounded by the Sac II sites in pHYB2#6 is in the correct orientation. Using primers MK25A28 and MK26A28 in the PCR screening acts as the negative control producing an approximate 180 bp fragment only in constructs containing the Sac II bounded fragment in the wrong orientation. pHYB2#6 sequence is determined using standard procedures.

pHYB2#6 has one mutation at position 378 which needed to be repaired to obtain a first quarter containing the desired sequence.

Plasmid p1HG#6 contains the desired sequence for the entire first half of the synthetic Bt gene. p1HG#6 is made from a 3.4 Kb Aat II\Nco I fragment of p1H1M5#2 ligated to a 500 bp Aat II\Nco I fragment from pHYB2#6.

To identify clones or partial clones of the synthetic gene which contain open reading frames, the kanamycin selection vector (described above) is used. The fourth quarter of the synthetic Bt gene is the first put into the kanamycin cassette. pKM74-4 contains the approximately 500 bp Apa I\Cla I fragment from plasmid BtP2 (which had been previously transformed into a dam- E. coli strain (PO-100) to be able to cut with Cla I), ligated to pUC:KM74 cut with Apa I\Cla I. Plasmid pKM74-4 displays kanamycin resistance but is later found to contain two substitution mutations at positions 1523 and 1634 (mutations are described above in the section on cloning the fourth quarter; they are substitutions, not deletions or insertions). The correct first half of the synthetic Bt gene from plasmid p1HG#6 is inserted into plasmid pKM74-4. The resulting plasmid, called pKm124, is made from the about 3.9 Kb Apa I\Bam HI fragment derived from pKM74-4 ligated to 1 Kb Apa I\Bam HI fragment from p1HG#6. pKm124 shows kanamycin resistance. This plasmid contains the first, second, and fourth quarters of the synthetic gene forming a single open reading frame.

The third quarter of the synthetic gene is next cloned into pKM124. The first functional clone, in plasmid pBt:Km#6, is a functional copy of the truncated synthetic cryIA(b) gene in the Km-cassette which displays kanamycin resistance but which contains deletion mutations between the third and fourth quarters. Plasmid pBt:Km#6 is made from the approximately 5 Kb Apa I\Nco I pKM124 vector fragment ligated to the approximately 500 bp Apa I\Nco I fragment from pQCN103 (pQCN103 contains a mismatch mutation at position 1326 which is repaired later). Contaminating nuclease activity appears to have deleted the Apa I site between the third and fourth quarters in pBt:Km#6. The Bt gene encoded by the synthetic gene in plasmid pBT:Km#6 has about 50-60 % of the native proteins' activity against ECB. The 2 Kb Sma I\Bam HI fragment from pBt:Km#6 is inserted into a 35S:expression cassette to make a plasmid called 35S:Bt6.

Two functional synthetic Bt clones, each with mutations, are initially obtained: plasmids pBT:KM#6 and pCIB4414. pCIB4414, which is 100% active in insect bioassays

against European corn borer compared with the native gene, contains substitution mutations in the third and fourth quarters at positions 1323, 1523, and 1634.

pCIB4414 is constructed from two plasmids, MG3.G4#18 and 1HG which is described above. MG3.G4#18 is obtained by cloning the Apa I/Kpn I fragment in plasmid Bt.P2#1 into pQCN103 (using those same restriction sites). This produces a plasmid containing the third and fourth quarters of the gene. The first half of the synthetic gene from plasmid 1HG is cut with Bam HI and Nco I and moved into MG3.G4#18 (containing the third and fourth quarters of the gene). The resulting plasmid, pCIB4414, contains a functional version of the synthetic gene. While being functional, the synthetic gene in this plasmid contains three errors; position 1326 (G substituted for a C), position 1523 (substitute A for a G), and at position 1634 (substitution of a T for a C).

The fourth quarter in pCIB4414 is replaced with a 354 bp fourth quarter Apa I\Bst E II fragment obtained from hybridizing, ligating, and restriction cleaving fourth quarter oligomers as described earlier, and isolating the fragment from a 2% Nusieve agarose gel. pCIB4408 is a synthetic Bt gene clone obtained by replacing the fourth quarter fragment in pCIB4414 with the hybridized fourth quarter fragment. To insert the CaMV 35S promoter in front of the synthetic Bt gene, pCIB4406 is made from a 4 Kb Eco NI\Kpn I fragment from plasmid p35SBt6 and 1.8 Kb Eco NI\Kpn I fragment from pCIB4408.

pCIB4406 is 100% active (as compared with the protein from the native gene) against ECB but contains the substitution mutation in the third quarter of the synthetic gene at position 1323 resulting in an amino acid substitution of a leucine for a phenylalanine. Plasmid pBS123#13 is used to repair this mutation.

The third quarter fragment in plasmid pBS123#13 is made from an approximately 479 bp hybridized oligomer generated fragment. Third quarter oligomers U15-U20 and L15-L21 are kinased, hybridized, and ligated as described above. PCR reactions are carried out as described above with primers P5(a) and P6(b) for 15 cycles. The PCR product is treated with proteinase K at a final concentration of about 50 μg\ml in an approximate 95 μl volume for 30 minutes at 37°C followed by 10 minutes at 65°C (Crowe et al., Nucleic Acid Research 19:184, 1991.) Subsequently, the product is phenol\chloroform extracted and ethanol precipitated using standard procedures before cutting with restriction enzymes Apa I and Nco I.

The approximate 450 bp Apa I\Nco I PCR fragment is ligated to the 3.8 Kb Apa I\Nco I vector fragment from p1HG#6 to make pBS123#13. Plasmid pBS123#13 contains the desired sequence for the third quarter of the maize optimized cryIA(b) gene from position 1319 at the Nsp I site through the Apa I site at position 1493. This 170 bp Nsp I\Apa I fragment from pBS123#13 is used in the fully active synthetic cryIA(b) gene in plasmid pCIB4418.

Western Blot Analysis:

Western blot analyses of various transformants are performed using crude extracts obtained from *E. coli* grown on selective plates. Using a toothpick, cultures are scraped from fresh plates containing the transformants of interest which have been grown overnight at 37°C. The positive control for expression of the Bt gene in *E. coli* was a construct called pCIB3069 which contains the native Bt-k gene fused with the plant expressible CaMV 35S promoter. pCIB3069 also contains the 35S promoter operably linked to the hygromycin resistance gene, 35S promoter, with Adh intron #1 operably linked to the GUS gene, and 35S promoter operably linked to a gene coding for the production of the native Bt cryIA(b) IP. A negative control of *E. coli* which does not contain a Bt gene is also included in the analyses. Cultures are resuspended in 100 μl of loading buffer containing 62 mM Tris-HCl pH 6.8, 1% SDS, 0.0025% bromophenol blue, 10% glycerol and 7.5% mercaptoethanol. After heating the mixtures at 95°C for 10 minutes, the preparations are sonicated for 1-3 seconds. The debris is centrifuged in a microfuge at room temperature for about 5 minutes and 10 to 15 μl of each sample is loaded onto an acrylamide gel with a 10% running gel below a 6% stacking gel (Laemmli, Nature 227;680-685(1970)). After electrophoresis overnight at 10 mAmps, proteins are transferred from the gel to an Immobilon membrane (Millipore). The transfer is done using an electrophoretic Blotting Unit (American BioNuclear, Emeryville, CA) in transfer buffer (20 mM Tris, 150 mM glycine, and 20% methanol) for 1.5 hours at 450 mAmps.

Buffers for western blotting included:

Blocking buffer: 2% Tween-20
30 mM Tris-HCl pH 10.2
150 mM NaCl

Wash buffer: 0.05% Tween-20
30 mM Tris-HCl pH 10.2
150 mM NaCl

Developing buffer: 100 mM Tris-HCl pH 9.6
100 mM NaCl
10 mM MgCl2

After transfer is complete, the membrane is incubated for about ten minutes in the blocking buffer. Three 15 minute washes with wash buffer are done before the first antibody treatment. The first antibody is an immunoaffinity purified rabbit or goat antibody prepared using the CryIA(b) protein as the antigen (Ciba-Geigy, RTP, N.C.; Rockland Inc., Gilbertsville, PA.; and Berkeley Antibody CO., Richmond, CA.). The cryIA(b) specific antibody is treated immediately before use with *E. coli* lysate from Bio-Rad in a 1 ml volume with 5 μg of antibody, 50 μl of E. coli lysate in the wash buffer solution. This mixture is incubated for 1 hour at room temperature before diluting it 1 to 30 for a final dilution of 1:6000 with wash buffer. Incubation of the membrane with the first antibody is at room temperature for 1.5 hours.

Three 10 minute washes are done between the 1st and 2nd antibody treatments. The second antibody is either rabbit anti-goat or goat anti-rabbit/alkaline phosphatase conjugate (Sigma, St. Louis, MO.). Incubation with the alkaline phosphatase conjugate is carried out at room temperature for one hour using a 1 to 6000 dilution in wash buffer. Six 10 minute washes are done between the second antibody treatment and developing the western blot. The western blot is developed in 100 ml of developing buffer with 440 μl of nitroblue tetrazolium in 70% dimethyl formamide (75 mg\ml), and 330 μl of 5-bromo-4-chloro-indolyl-phosphate in 100% dimethyl formamide (50 mg\ml). After developing for 15 to 30 minutes, the membrane is washed in water and air dried.

EXAMPLE 4: Construction Of Transformation Vectors

Construction of pCIB710 and derivatives.

CaMV 35S Promoter Cassette Plasmids pCIB709 and pCIB710 are constructed as shown in Rothstein et al., Gene 53:153-161 (1987). pCIB710 contains CaMV promoter and transcription termination sequences for the 35S RNA transcript (Covey et al., Nucl. Acids. Res., 9:6735-6747 (1981)). A 1149 bp BglII restriction fragment of CaMV DNA (bp 6494-7643 in Hohn et al., Current Topics in Microbiology and Immunology, 96:194-220 and Appendices A to G (1982)) is isolated from CaMV DNA by preparative agarose gel electrophoresis as described earlier The fragment is mixed with BamHI-cleaved plasmid pUC19 DNA, treated with T4 DNA ligase, and transformed into *E. coli.* (Note the BamHI restriction site in the resulting plasmid is destroyed by ligation of the Bg1II cohesive ends to the BamHI cohesive ends.)

The resulting plasmid, called pUC19/35S, is then used in oligonucleotide-directed in-vitro mutagenesis to insert the BamHI recognition sequence GGATCC immediately following CaMV nucleotide 7483 in the Hohn reference. The resulting plasmid, pCIB710, contains the CaMV 35S promoter region and transcription termination region separated by a BamHI restriction site. DNA sequences inserted into this BamHI site will be expressed in plants by these CaMV transcription regulation sequences. (Also note that pCIB710 does not contain any ATG translation initiation codons between the start of transcription and the BamHI site).

pCIB710 is modified to produce pCIB709 by inserting a Bam HI fragment containing the coding sequence for hygromycin phosphotransferase from pLG90 (Rothstein et al., Gene, 53:153-161 (1987)) in the Bam HI site.

pCIB709 is modified to produce pCIB996 by removing the ATG just upstream from the initiation codon of the hygromycin phosphotranserase gene using standard mutagenesis techniques while inserting a Bgl II restriction site at this location. The resulting plasmid, pCIB996, is further modified to remove the Bam HI, Sma I and Bgl II sites in the 5' untranslated leader region located 5' of the initiation codon for the initiation codon. The result is a change of DNA base sequence from -TATAAGGATC CCGGGGGCA AGATCTGAGA TATG (SEQ ID NO:59)-Hyg to -TATAAGGATC TGAGATATG (SEQ ID NO:59 with nucleotides 11-24 deleted)-Hyg. The resulting plasmid is known as pCIB3073.

Alternatively, pCIB710 is modified to produce pCIB900, by inserting the Bam HI - Bcl I fragment of pCIB10/35SBt, which contains the 645 amino acid Bt coding sequence, described in Part C4 below, into the Bam HI site of pCIB710 to create pCIB710/35SBt. To introduce an antibiotic resistance marker, pCIB709 is cut with Sal I, a Kpn I/Sal I adaptor is ligated and the resulting ligation product is cut with Kpn I. The Kpn fragment of pCIB709 containing the 35S/hygromycin resistance gene is inserted into the Kpn I site of pCIB710/35SBt to produce pCIB900.

Genes useful as the selectable marker gene include the hygromycin resistance gene described in Rothstein et al., Gene 53: 153-161 (1987). The hygromycin gene described in this reference is moved into a pUC plasmid such as pCIB710 or pCIB709 and the "extra" ATG upstream from the hygromycin phosphotransferase coding sequence is removed to create pCIB996. This modified pCIB996 gene is further modified to remove a BglII, BamHI and SmaI sites from the 5' region of the gene using standard techniques of molecular biology to make pCIB3073.

pCIB932 is a pUC19-based plasmid containing the chimeric gene Pep-C:promoter\Bt\Pep-C:terminator. It is composed of fragments derived from pPEP-10, a HindIII subclone of a genomic clone, H1-lambda-14, PNAS USA, 83:2884-2888 (1986), of the maize gene encoding the PEP carboxylase enzyme active in photosynthesis, and from pCIB930, which is a BamHI fragment containing the 645 amino acid truncated form of the the cryIAb endotoxin gene in the BamHI site of pUC18.

The 2.6 kb EcoRI-XhoI fragment from pPEP-10, containing the polyA addition site from the PEP carboxylase gene, is isolated and digested with PstI and HincII. The restriction digest is ligated with PstI/HincII digested pUC18, transformed into *E. coli* and transformants screened for those containing a 412 bp PstI-HincII insert in pUC18 and the insert verified by sequencing. The resulting plasmid is called pCIB931.

The nuclear gene encoding the phosphoenolpyruvate carboxylase isozyme ("Pep-C") is described in Hudspeth et al., Plant Molecular Biology, 12: 579-589 (1989). pCIB932 is constructed by the ligation of three fragments. The first fragment, containing the PEP-C transcription terminator, is produced by digesting pCIB931 to completion with HindIII, partially with SphI and the 3098 bp fragment isolated. The second fragment, containing the Bt endotoxin

coding sequence, is produced by digesting pCIB930 with NcoI and SphI and isolating the 1950 bp fragment. The third fragment, containing the PEP-C promoter, is produced by digesting pPEP-10 to completion with HindIII, partially with NcoI and isolating the 2.3 kb fragment. The ligation mix is transformed into *E. coli*, transformants with the correct insertion identified and the insert verified by sequencing.

pCIB932 is cut with PvuII to generate a 4.9 Kb fragment containing the maize Pep-C:promoter\Bt\Pep-C:terminator and purified on a 1% LGT agarose gel in 1X TAE. The linearized pCIB3079 vector and the 4.9 Kb insert from pCIB932 are ligated using T4 DNA ligase in LGT to make pCIB4401. pCIB4401 is a maize transformation vector containing the chimeric genes: 35S:promoter\PAT\35S:terminator, Pep-C:promoter\Bt\Pep-C: terminator, and 35S:promoter\AdhI #1 intron\GUS\35S: terminator.

Construction of pCIB246 (35S-GUS-35S)

A CaMV 35S promoter cassette, pCIB246, is constructed as follows.

The DdeI restriction site at nucleotide position 7482 of the CaMV genome (Franck et al., Cell, 21:285-294 (1980)) is modified by insertion of a 48 bp oligonucleotide containing several restriction enzyme sites including an NcoI (CCATGG) site, a SalI (GTCGAC) site, and an SstI (GAGCTC) site. This altered CaMV 35S promoter is inserted into a pUC19 vector that had been modified to destroy the vector's SstI and SalI sites. Thus, the CaMV 35S promoter of pCIB1500 contains unique SstI and SalI sites for cloning.

pCIB1500 is digested with SstI/NcoI and ligated with the GUS gene obtained from pBI221 (Clontech Laboratories, Inc., Palo Alto, CA). The NcoI site is fused to the GUS gene such that the ATG of the NcoI site functions as the start codon for the translation of the GUS gene. The CaMV 35S polyadenylation and termination signals are used for the 3' end of the chimeric gene.

Construction of pCIB3069 (35S-Adh1-GUS-35S)

pCIB246 is modified by adding the maize alcohol dehydrogenase gene Adh1 intron number 1 (Adh1) (Dennis et al., Nucleic Acids Research, 12:3983-4000 (1984)) into the Sal I site of pCIB246 to produce plasmid pCIB3007. The Adh1 intron is excised from the maize Adh1 gene as a Bal I/Pst I fragment and subcloned into pUC18 that was cut with Sma I/Pst I to make a plasmid called Adh 1026. Adh 1026 is cut with Pvu II/Sac II, the fragments are made blunt

ended with T4 DNA polymerase, Sal I linkers are added using standard procedures and a fragment of about 560 bp is recovered from a 3 % NuSeive gel and ligated into Sal I cut/phosphatase treated pUC18. The Sal I linkered Adh intron #1 in the resulting plasmid is cut out with Sal I, gel purified, and ligated into Sal I cut/ phosphatase treated pCIB246 to make plasmid pCIB3007.

pCIB3007 is cut with PstI and the ends made blunt by using T4 DNA polymerase (NEW England Biolabs) according to the suppliers' specifications. The resulting blunt ended molecules are cut with Sph I and the approximately 5.8 Kb fragment with one blunt end and one Sph I end is purified on a low gelling temperature (LGT) agarose gel using standard procedures. pCIB900 is cut with Sma I/Sph I and the fragment containing the 35S/Bt gene is purified on a LGT agarose gel. The two gel purified fragments are ligated in LGT agarose using T4 DNA ligase according to standard conditions. The resulting ligated fragments are transformed into E. *coli* using standard procedures and the resulting plasmid is called pCIB3062. There are two versions of pCIB3062. pCIB3062#1 has a Sma I site regenerated where the Sma I site and the T4 polymerase blunted ends are ligated. This most likely results from the T4 polymerase nibbling a few base pairs from the Pst I site during the blunting reaction. pCIB3062#3 does not have this SmaI site.

pCIB3062#3 is cut with KpnI and made blunt-ended using T4 DNA polymerase, and subsequently cut with Pvu II to yield a 6.4 Kb fragment with blunt ends containing the 35S/GUS and 35S/Bt genes. This blunt-end fragment is ligated into Sma I cut pCIB3073 to produce pCIB3063 or pCIB3069. pCIB3069 contains the same fragment used to make pCIB3063, but the chimeric genes in pCIB3069 are all in the same relative orientation, unlike those in pCIB3063. These plasmids contain a) a 35S promoter operably linked to the hygromycin resistance gene; b) a 35S promoter, with Adh intron #1, operably linked to the GUS gene; and c) a 35S promoter operably linked to a gene coding for the production of the synthetic cryIA(b) insecticidal protein from *Bacillus thuringiensis*, as described above.

GUS Assays:

GUS assays are done essentially as described in Jefferson, Plant Mol. Bio. Reporter, 5:387-405 (1987). As shown above, plasmid pCIB246 contains a CaMV 35S promoter fused with the GUS gene. The 5' untranslated leader of this chimeric gene contains a copy of the maize

Adh1 intron #1. It is used here as a transformation control. Although the same amount of pCIB246 is added to each transformation, the calculated activity varied among Bt constructs tested. The values reported below are averages of 3 replicates. pCIB4407 was tested twice.

pCIB3069	28 nM MU/ug/min
pCIB4407	0.7 nM MU/ug/min, 2.3 nM MU/ug/min

EXAMPLE 5A: Assay Of Synthetic cryIA(b) Gene For Insecticidal Activity Against European Corn Borer

The synthetic cryIA(b) gene in pCIB4414 in *E. coli* is assayed for insecticidal activity against European corn borer according to the following protocol.

Molten artifical insect diet is poured into a 60 mm Gellman snap-cap petri dish. After solidification, *E. coli* cells, suspended in 0.1% Triton X-100, are spread over the surface at a concentration of 3 X 107 cells/cm2. The plates are air dried. Ten first instar European corn borer, *Ostrinia nubilalis*, which are less than 12 hours old are then placed onto the diet surface. The test is incubated at 30 C in complete darkness for 2-5 days. At the end of the test percent mortality is recorded. A positive clone has been defined as one giving 50% or higher mortality when control *E. coli* cells give 0-10% background mortality.

For comparison, the native cryIA(b) gene in pCIB3069 is tested at the same concentration. Clones are tested at 3 x 10^7 cells/cm^2 diet; 20 insects per clone.

The following results are observed:

Clone	Percent Mortality
Control	0
pCIB3069	100
pCIB4414	100

These results indicate that the insecticidal crystal protein produced by the synthetic cryIA(b) gene demonstrates activity against European corn borer comparable to that of the IP

produced by the native cryIA(b). Other plasmids containing a synthetic cryIA(b) gene were assayed in a similar manner.

EXAMPLE 5B: Assay Of CRYIA(b) Protein For Insecticidal Activity Against Sugarcane Borer.

CryIA(b) was expressed in *E. coli* and assayed for insecticidal activity against Sugarcane borer (*Diatrea saccharalis*) according to the same protocol used for European corn borer, described immediately above. The results are summarized in the Table.

TABLE

SUGARCANE BORER ASSAY WITH Bt PROTEIN FROM *E. COLI*

Protein Concentration (ng/g)	Percent Mortality CryIA(b)
10	0
25	0
50	7
100	13
250	40
500	53
1000	80
LC50	380
95% Cl	249-646

The results indicate that the insecticidal protein produced by a maize optimized Bt gene is effective against Sugarcane borer. The upper concentrations of CryIA(b) protein, 250 ng/g-1000 ng/g, are achievable in transgenic maize plants produced in accordance with the instant invention.

EXAMPLE 6: Maize Protoplast Isolation And Transformation With The Synthetic Bt Gene

Expression of the synthetic Bt gene is assayed in transiently transformed maize protoplasts.

Protoplast Isolation Procedure:

1. The contents of 10 two day old maize 2717 Line 6 suspension cultures are pipetted into 50 ml sterile tubes and allowed to settle. All culture media is then removed and discarded.

2. Cells (3-5 ml Packed Cell Volume) are resuspended in 30 ml protoplast enzyme solution. Recipe follows:

3% Cellulase RS

1% Macerozyme R10 in KMC Buffer

KMC Buffer (recipe for 1 liter)

KCl	8.65 g
$MgCl_2$-$6H_2O$	16.47 g
$CaCl_2$-$2H_2O$	12.50 g
MES	5.0 g

pH 5.6, filter sterilize

3. Mix cells well and aliquot into 100x25 mm petri dishes, about 15 ml per plate. Shake on a gyratory shaker for 4 hours to digest.

4. Pipette 10 ml KMC through each 100 micron sieve to be used. Filter contents of dishes through sieve. Wash sieve with an equal volume KMC.

5. Pipette sieved protoplasts carefully into 50 ml tubes and spin in a Beckman TJ-6 centrifuge for 10 minutes at 1000 rpm (500 x g).

6. Remove supernatant and resuspend pellet carefully in 10 ml KMC. Combine contents of 3 tubes into one and bring volume to 50 ml with KMC.

7. Spin and wash again by repeating the above step.

8. Resuspend all washed protoplasts in 50 ml KMC. Count in a hemocytometer. Spin protoplasts and resuspend at 8 x 10^6/ml in resuspending buffer (RS Buffer).

RS Buffer (recipe for 500 ml)

mannitol	27.33 g
$CaCl_2$ (0.1 M stock)	75 ml
MES	0.5 g

pH 5.8, filter sterilize

Protoplast Transformation Procedure:

1. Aliquot 50 μg plasmid DNA (Bt IP constructs, both synthetic (pCIB4407) and native (pCIB3069)) to 15 ml polystyrene culture tubes. Also aliquot 25 μg GUS-containing plasmid DNA (which does not contain Bt IP (pCIB246) to all tubes. 3 replications are used per construct to be tested, with 1 rep containing no DNA as a control.

Bt constructs:	GUS construct:
pCIB3069	pCIB246
pCIB4407	

2. Gently mix protoplasts well and aliquot 0.5 ml per tube.

3. Add 0.5 ml PEG-40 to each tube.

PEG-40:

0.4 M mannitol

0.1 M $Ca(NO_3)_2$-$4H_2O$

pH 8.0, filter sterilize

4. Mix gently to combine protoplasts with PEG. Wait 30 minutes.

5. Sequentially add 1 ml, 2 ml, and 5 ml W5 solution at 5 minute intervals.

W5 Solution:

154 mM NaCl

125 mM $CaCl_2$-H_2O

5 mM KCl

5 mM glucose

pH 7.0, filter sterilize

6. Spin for 10 minutes in a Beckman TJ-6 centrifuge at about 1000 rpm (500g). Remove supernatant.

7. Gently resuspend pellet in 1.5 ml FW media and plate carefully in 35x10 mm petri dishes.

FW media (recipe for 1 liter):

MS salts 4.3 g

200X B5 vits. 5 ml

sucrose 30 g

proline 1.5 g

mannitol 54 g

2,4 D 3 mg

pH 5.7, filter sterilize

8. Incubate overnight in the dark at room temperature.

9. Perform GUS assays, insect bioassays, and ELISA's on protoplast extracts as described below.

EXAMPLE 7: Construction Of A Full-Length Synthetic Maize Optimized cryIA(b) Gene

SEQ ID NO:4 shows the synthetic maize optimized sequence encoding the full-length cryIA(b) insecticidal protein from *B. thuringiensis*. The truncated version described above represents the first approximately 2 Kb of this gene. The remainder of the full-length gene is cloned using the procedures described above. Briefly, this procedure entails synthesizing DNA oligomers of 40 to 90 NT in length, typically using 80 mers as an average size. The oligomers are purified using standard procedures of HPLC or recovery from a polyacrylamide gel. Purified oligomers are kinased and hybridized to form fragments of about 500 bp. The hybridized oligomers can be amplified using PCR under standard conditions. The 500 bp fragments, either directly from hybridizations, from PCR amplification, or recovered from agarose gels after either hybridization or PCR amplification, are then cloned into a plasmid and transformed into *E. coli* using standard procedures. Recombinant plasmids containing the desired inserts are identified, as described above, using PCR and/or standard miniscreen procedures. Inserts that appear correct based upon their PCR and/or restriction enzyme profile are then sequenced to identify those clones containing the desired open reading frame. The fragments are then ligated together with the approximately 2 Kb synthetic sequence described in Example 2 to produce a full-length maize optimized synthetic cryIA(b) gene useful for expression of high levels of CryIA(b) protein in maize.

G+C Content of native and synthetic Bt genes:

Full-length native 38.8%

Truncated native	37.2%
Full-length synthetic	64.8%
Truncated synthetic	64.6%

% homology of the final truncated version of the Bt gene relative to a "pure" maize codon usage gene: 98.25%

EXAMPLE 8: Construction of a plant expressible, full-length, hybrid partially maize optimized cryIA(b) gene.

pCIB4434 contains a full length CryIA(b) gene (SEQ ID NO:8) comprised of about 2 Kb of the synthetic maize optimized cryIA(b) gene with the remainder (COOH terminal encoding portion) of the gene derived from the native gene. Thus, the coding region is a chimera between the synthetic gene and the native gene, but the resulting protein is identical to the native cryIA(b) protein. The synthetic region is from nucleotide 1-1938 (amino acids 1 to 646) and the native coding sequence is from nucleotide 1939-3468 (amino acids 647 to 1155). The sequence of this gene is set forth in Fig. 7. A map of pCIB4434 is shown in Fig. 8.

The following oligos were designed to make pCIB4434:

KE134A28 = 5'-CGTGACCGAC TACCACATCG ATCAAGTATC CAATTTAGTT GAGT-3' (SEQ ID NO:60)

KE135A28 = 5'-ACTCAACTAA ATTGGATACT TGATCGATGT GGTAGTCGGTC ACG-3' (SEQ ID NO:61)

KE136A28 = 5'-GCAGATCTGA GCTCTTAGGT ACCCAATAGC GTAACGT-3' (SEQ ID NO:62)

KE137A28 = 5'-GCTGATTATG CATCAGCCTAT-3' (SEQ ID NO:63)

KE138A28 = 5'-GCAGATCTGA GCTCTTATTC CTCCATAAGA AGTAATTC-3' (SEQ ID NO:64)

MK05A28 = 5'-CAAAGGTACC CAATAGCGTA ACG-3' (SEQ ID NO:65)

MK35A28 = 5'-AACGAGGTGT ACATCGACCG-3' (SEQ ID NO:66)

pCIB4434 is made using a four-way ligation with a 5.7 kb fragment from pCIB4418, a 346 bp Bst E II\Kpn I PCR-generated synthetic:native fusion fragment, a 108 bp Kpn I\Nsi I native CryIA(b) fragment from pCIB1315, and a 224 bp Nsi I\Bgl II PCR-generated fragment. Standard conditions for ligation and transformation are as described previously.

A synthetic:native gene fusion fragment is made in two steps using PCR. The first 253 bp of the PCR fusion fragment is made using 100 pmols of oligos KE134A28 and MK04A28 with approximately 200 ng of native cryIA(b) template in a 100 ul volume with 200 nm of each dNTP, 1 X PCR buffer (Perkin Elmer Cetus), 20 % glycerol, and 5 units of Taq polymerase (Perkin Elmer Cetus). The PCR reaction is run with the following parameters: 1 minute at 94°C, 1 minute at 55°C, 45 seconds at 72°C, with extension 3 for 3 seconds for 25 cycles. A fraction (1 %) of this first PCR reaction is used as a template along with 200 ng of the synthetic cryIA(b) DNA to make the complete 351 bp synthetic:native fusion fragment. Oligos used as PCR primers in this second PCR reaction are 50 pmols of MK35A28, 50 pmols of MK04A28, and 25 pmols of KE135A28. The PCR reaction mix and parameters are the same as those listed above. The resultant 351 bp synthetic:native fusion fragment is treated with Proteinase K at 50 ug\ml total concentration and phenol\chloroform extraction followed by ethanol precipitation before cutting with Bst E II\Kpn I using standard conditions.

The 224 bp Nsi I\Bgl II PCR fragment used in making pCIB4434 is made using 100 pmols of oligos KE137A28 and KE138A28 and 200 ng of the native cryIA(b) gene as template in 100 ul volume with the same PCR reaction mix and parameters as listed above. The 230 bp PCR native cryIA(b) fragment is treated with Proteinase K, phenol\chloroform extracted, and ethanol precipitated as described above, before cutting with Nsi I\Bgl II.

pCIB4434 was transformed into maize protoplasts as described above. Line 6 2717 protoplasts were used with pCIB4434 and pCIB4419 as a control for comparison. The results are shown below:

	ng Bt/mg protein
4419(35S)	14,400 ± 2,100
4434(full-length)	2,200 ± 900

Background = 13 ng Bt/mg protein for untransformed protoplasts

The results indicate that pCIB4434 expresses at a level of about 15% of pCIB4419.

Western blot analysis shows at least one-third of the cryIA(b) protein produced by pCIB4434 in this system is about 130 kD in size. Therefore, a significant amount of full-length cryIA(b) protein is produced in maize cells from the expression of pCIB4434.

EXAMPLE 8A: Construction of a full-length, cryIA(b) genes encoding a temperature-stable cryIA(b) protein.

Constructs pCIB5511-5515, each containing a full-length, cryIA(b) gene are described below. In these sequences, the 26 amino acid deletion between amino acids 793 and 794, KCGEPNRCAPHLEWNPDLDCSCRDGE (see: SEQ ID NOS:8, 10, 12, 14, 16), present in cryIA(a) and cryIA(c) but not in cryIA(b), has been repaired. The gene in pCIB5513 is synthetic; the other four genes are hybrids, and thus are partially maize optimized.

Construction of pCIB5511

This plasmid is a derivative of pCIB4434. A map of pCIB5511 is shown in Fig. 10. A 435 bp segment of DNA between bp 2165 and 2590 was constructed by hybridization of synthetic oligomers designed to represent the upper and lower strand as described above for the construction of the truncated cryIA(b) gene. This segment of synthetic DNA is synthesized using standard techniques known in the art and includes the 26 amino acid deletion found to occur naturally in the cryIA(b) protein in *Bacillus thuringiensis* kurstaki HD-1. The entire inserted segment of DNA uses maize optimized codon preferences to encode amino acids. The 26 amino acids used to repair the naturally occurring deletion are contained within this fragment. They are

inserted starting at position 2387 between the KpnI site at nt 2170 and the XbaI site at nt 2508 (2586 in pCIB5511) of pCIB4434. pCIB5511 is constructed via a three way ligation using a 3.2 Kb fragment obtained by restriction digestion of pCIB4434 with SphI and KpnI, a 3.8 Kb fragment obtained by digestion of pCIB4434 with SphI and XbaI, and a 416 bp fragment obtained by digestion of the synthetic DNA described above, with KpnI and XbaI. Enzymatic reactions are carried out under standard conditions. After ligation, the DNA mixture is transformed into competent *E. coli* cells using standard procedures. Transformants are selected on L-agar containing 100 μg/ml ampicillin. Plasmids in transformants are characterized using standard mini-screen procedures. The sequence of the repaired cryIA(b) gene encoding the cryIA(b) temperature (heat) stable protein is set forth in Fig. 9 (SEQ ID NO:10).

Construction of pCIB5512

This plasmid construct is a derivative of pCIB4434. A map of pCIB5512 is shown in Fig. 12. DNA to repair the 26 amino acid deletion is prepared using standard techniques of DNA synthesis and enzymatic reaction. Three double stranded DNA cassettes, pGFcas1, pGFcas2 and pGFcas3, each about 300 bp in size, are prepared. These cassettes are designed to contain the maize optimized codons while maintaining 100% amino acid identity with the insecticidal protein. These cassettes are used to replace the region between restriction site BstEII at position 1824 and XbaI at position 2508 and include the insertion of the additional 78 bp which encode the missing 26 amino acids (described above for pCIB5511 in pCIB4434). Each of these cassettes is cloned into the EcoRV site of the vector Bluescript (Stratagene) by standard techniques. The three cassettes are designed to contain overlapping restriction sites. Cassette 1 has restriction sites BstEII at the 5' end and EcoRV at the 3' end: cassette 2 has EcoRV at the 5' end and ClaI at the 3' end and cassette 3 has ClaI at the 5' end and Xba I at the 3' end. They are cloned individually in Bluescript and the the complete 762 bp fragment is subsequently assembled by ligation using standard techniques. pCIB5512 is assembled using this 762 bp fragment and ligating it with a 6.65 Kb fragment obtained by a complete digestion of pCIB4434 with BstEII and a partial digestion with XbaI. Alternatively, a four way ligation using the same vector and the three cassettes digested with the specific enzymes can be employed. Enzymatic reactions are carried out under standard conditions. After ligation, the DNA mixture is transformed into competent *E. coli* cells using standard procedures. Transformants are selected

on L-agar containing 100 μg/ml ampicillin. Plasmids in transformants are characterized using standard mini-screen procedures. The resulting plasmid is pCIB5512. The sequence of the repaired cryIA(b) gene is illustrated in Fig. 11 (SEQ ID NO:12). This repaired cryIA(b) differs from that carried in pCIB5511 in that a larger region of the cryIA(b) coding region is optimized for maize expression by using maize preferred codons.

Construction of pCIB5513

This plasmid contains a repaired cryIA(b) gene derived from pCIB5512. A map of pCIB5513 is shown in Fig. 14. The region 3' from the XbaI site at position 2586 to the end of the gene (BglII site at position 3572) is replaced entirely with maize optimized codons. This region is synthesized, using standard techniques of DNA synthesis and enzymatic reaction, well known in the art, as four double stranded DNA cassettes (cassettes # 4,5 ,6 ,7). Adjacent cassettes have overlapping restriction sites to facilitate assembly between cassettes. These are XbaI and XhoI at the 5' and 3' ends of cassette 4; XhoI and SacI at the 5' and 3' ends, respectively, of cassette 5; SacI and BstXI at the 5' and 3' ends, respectively, of cassette 6; and BstXI and BglII at the 5' and 3' ends, respectively, of cassette 7. As described for pCIB5512, the cassettes are cloned into the blunt-end EcoRV site of the Bluescript vector (Stratagene) and the full-length "repaired" cryIA(b) gene cloned either by sequential assembly of the above cassettes in Bluescript followed by ligation of the complete 967 bp synthetic region with a 6448 bp fragment obtained by a complete digestion of pCIB5512 with BglII and a partial digestion with XbaI. Alternately, the plasmid containing the full-length genes is obtained by a 5-way ligation of each of the four cassettes (after cleavage with the appropriate enzymes) and the same vector as above. The sequence of the full-length, "repaired" cryIA(b) gene is set forth in Fig. 13 (SEQ ID NO:14). The protein encoded by the various synthetic and synthetic/native coding region chimeras encode the same protein. This protein is the heat-stable version of cryIA(b) produced by repairing the naturally occurring 26 amino acid deletion found in the cryIA(b) gene from *Bacillus thuringiensis* kurstaki HD-1 when the homologous region is compared with either cryIA(a) or cryIA(c) *Bacillus thuringiensis* delta-endotoxins.

Construction of pCIB5514

This plasmid is a derivative of pCIB4434. A map of pCIB5514 is shown in Fig. 16. It is made using synthetic DNA cassette #3 (see above) which contains a maize optimized sequence

of the region between the ClaI site (position 2396) found in the 26 amino acid thermostable region and the XbaI site at position 2508 in pCIB4434 (2586 in pCIB5511). The region between nt 2113 of pCIB4434 and the junction of the thermostable region is PCR amplified by using pCIB4434 as template with the following primers:

forward: 5'GCACCGATATCACCATCCAAGGAGGCGATGACGTATTCAAAG-3' (SEQ ID NO:67)

reverse:

5'-AGCGCATCGATTCGGCTCCCCGCACTTGCCGATTGGACTTGGGGCTGAAAG-3' (SEQ ID NO:68).

The PCR product is then digested with restriction enzymes KpnI and ClaI and ligated in a four part reaction with a 189 bp fragment obtained by digestion of cassette 3 with ClaI and XbaI, a 3.2 Kb fragment of pCIB4434 digested with SphI and KpnI, and a 3.8 Kb fragment of pCIB4434 obtained by digestion with SphI and Xba. Enzymatic reactions are carried out under standard conditions. The ligation product is transformed into competent *E coli* cells, selected with ampicillin and screened using standard procedures described above. The sequence of the repaired cryIA(b) gene contained in pCIB5514 is shown in Fig. 15 (SEQ ID NO:16).

pCIB4434 was modified by adding the 78bp Geiser thermostable element (Geiser TSE), described above, between the Kpn I site (2170 bp) and the Xba I site (2508 bp) in the native Btk region. The exact insertion site starts at the nucleotide #2379. The region containing the Geiser TSE was amplified by two sets of PCR reactions, i.e. the Kpn I - Geiser TSE fragment and the Geiser TSE - Xba I fragment.

PCR primer#1: (Kpn I site)

5' - ATTACGTTAC GCTATTGGGT ACCTTTGATG - 3' (SEQ ID NO:69)

PCR primer#2: (Geiser TSE bottom)

5' - TCCCCGTCCC TGCAGCTGCA GTCTAGGTCC GGGTTCCACT CCAGGTGCGG AGCGCATCGA TTCGGCTCCC CGCACTTGCC GATTGGACTT GGGGCTGA - 3' (SEQ ID NO:70)

PCR primer#3: (Geiser TSE top)

5' - CAAGTGCGGG GAGCCGAATC GATGCGCTCC GCACCTGGAG TGGAACCCGG ACCTAGACTG CAGCTGCAGG GACGGGGAAA AATGTGCCCA TCATTCCC - 3' (SEQ ID NO:71)

PCR primer#4: (Xba I site)

5' - TGGTTTCTCT TCGAGAAATT CTAGATTTCC - 3' (SEQ ID NO:72)

After the amplification, the PCR fragments were digested with (Kpn I + Cla I) and (Cla I + Xba I), respectively. These two fragments were ligated to the Kpn I and Xba I digested pCIB4434. The resulting construct pCIB5515 is pCIB4434 with a Geiser TSE and an extra Cla I site flanked by Kpn I and Xba I. A map of pCIB5515 is illustrated in Fig. 38. The cryIA(b) gene contained herein, which encodes a temperature stable cryIA(b) protein, is shown in Fig. 37 (SEQ ID NO:27).

Examples 9-20 set forth below are directed to the isolation and characterization of a pith-preferred promoter.

EXAMPLE 9: RNA Isolation and Northern Blots

All RNA was isolated from plants grown under greenhouse conditions. Total RNA was isolated as described in Kramer et al., Plant Physiol., 90:1214-1220 (1990) from the following tissues of Funk maize line 5N984: 8, 11, 15, 25, 35, 40, and 60 day old green leaves; 8, 11, 15, 25, 35, 39, 46, 60 and 70 day old pith; 60 and 70 day old brace roots from Funk maize line 5N984; 60 and 70 day 5N984 sheath and ear stock. RNA was also isolated from 14 day 211D roots and from developing seed at weekly intervals for weeks one through five post-pollenation. Poly A+ RNA was isolated using oligo-dT as described by Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd ed.), 1989, and Northern blots were carried out, also as per Sambrook et al. using either total RNA (30 μg) or poly A+ RNA (2-10 μg). After electrophoresis, RNA was blotted onto Nitroplus 2000 membranes (Micron Separations Inc). The RNA was linked to

the filter using the Stratalinker (Stratagene) at 0.2 mJoules. The northerns were probed with the 1200 bp EcoRI pith (TRpA) 8-2 cDNA fragment, isolated by using 0.8% low melting temperature agarose in a TBE buffer system. Northerns were hybridized and washed and the filters exposed to film as described in Isolation of cDNA clones.

EXAMPLE 10. Isolation of cDNA Clones

First strand cDNA synthesis was carried out using the BRL AMV reverse transcriptase system I using conditions specified by the supplier (Life Technologies, Inc., Gaithersburg, MD). Specifically, 25 μl reactions containing 50 mM Tris-HCl pH 8.3, 20 mM KCl, 1 mM DTT, 6 mM MgCl2, 1 mM each of each dNTP, 0.1 mM oligo (dT)12-18, 2 μg pith poly(A+) RNA, 100 μg/ml BSA, 50 μg/ml actinomycin D, 8 units placental RNase inhibitor, 1 μl (10 mM Ci/ml) 32P dCTP >3000 mCi/mM as tracer, and 30 units AMV reverse transcriptase were incubated at 42°C for 30 min. Additional KCl was added to a concentration of 50 mM and incubation continued a further 30 min. at 42°C. KCl was added again to yield a final concentration of 100 mM. Additional AMV reverse transcriptase reaction buffer was added to maintain starting concentrations of the other components plus an additional 10 units, and the incubation continued at 42°C for another 30 min. Second strand synthesis was completed using the Riboclone cDNA synthesis system with Eco RI linkers (Promega, Madison, WI). Double stranded cDNA was sized on an 1% agarose gel using Tris-borate-EDTA buffer as disclosed in Sambrook et al., and showed an average size of about 1.2 Kb. The cDNA was size fractionated using NA45 DEAE membrane so as to retain those molecules of about 1000 bp or larger using conditions specified by the supplier (Schleicher and Schuell). Size fractionated cDNA was ligated into the Lambda ZapII vector (Stratagene, La Jolla, CA) and packaged into lambda particles using Gigapack II Plus (Stratagene, La Jolla, CA). The unamplified library had a titer of 315,000 pfu while the amplified library had a titer of 3.5 billion/ml using PLK-F' cells.

Recombinant phage were plated at a density of 5000 pfu on 150 X 15mm L-agar plates. A total of 50,000 phage were screened using duplicate lifts from each plate and probes of first strand cDNA generated from either pith derived mRNA or seed derived mRNA. The lifts were done as described in Sambrook et al. using nitrocellulose filters. DNA was fixed to the filters by UV crosslinking using a Stratalinker (Stratagene, La Jolla, CA) at 0.2 mJoule. Prehybridization

and hybridization of the filter were carried out in a solution of 10X Denhardts solution, 150 μg/ml sheared salmon sperm DNA, 1% SDS, 50 mM sodium phosphate pH 7, 5 mM EDTA, 6X SSC, 0.05% sodium pyrophosphate. Prehybridization was at 62°C for 4 hours and hybridization was at 62°C for 18 hours (overnight) with 1 million cpm/ml in a volume of 40 ml. Filters were washed in 500 ml of 2X SSC, 0.5% SDS at room temperature for 15 min. then at 63°C in 0.1X SSC, 0.5% SDS for 30 min. for each wash. Radiolabeled DNA probes were made using a BRL random prime labeling system and unincorporated counts removed using Nick Columns (Pharmacia). Filters were exposed overnight to Kodak X-Omat AR X-ray film with (DuPont) Cronex Lightning Plus intensifying screens at -80°C. Plaques showing hybridization with the pith-derived probe and not the seed-derived probe were plaque purified for further characterization.

EXAMPLE 11. Isolation of Genomic Clones

Genomic DNA from Funk inbred maize line 211D was isolated as described by Shure et al., Cell, 35:225-233 (1988). The DNA was partially digested with Sau 3A and subsequently size fractionated on 10-40% sucrose gradients centrifuged in a Beckman SW40 rotor at 22,000 rpm for 20 hours at 20°C. Fractions in the range of 9-23 Kb were pooled and ethanol precipitated. Lambda Dash II (Stratagene) cut with Bam HI was used as described by the supplier. The library was screened unamplified and a total of 300,000 pfu were screened using the conditions described above. The library was probed using pith- specific (TrpA) cDNA clone 8-2, pCIB5600 which was identified in the differential screen of the cDNA library. Isolated clones were plaque purified and a large scale phage preparation was made using Lambdasorb (Promega) as described by the supplier. Isolated genomic clones were digested with Eco RI and the 4.8 kb EcoRI fragment was subcloned into Bluescript vector (Stratagene).

EXAMPLE 12. DNA Sequence and Computer Analysis

Nucleotide sequencing was performed using the dideoxy chain-termination method disclosed in Sanger et al., PNAS,74:5463-5467 (1977). Sequencing primers were synthesized on an Applied Biosystems model 380B DNA synthesizer using standard conditions. Sequencing reactions were carried out using the Sequenase system (US Biochemical Corp.). Gel analysis

was performed on 40 cm gels of 6% polyacrylamide with 7 M urea in Tris-Borate-EDTA buffer (BRL Gel-Mix 6). Analysis of sequences and comparison with sequences in GenBank were done using the U. of Wisconsin Genetic Computer Group Sequence Analysis Software (UWGCG).

EXAMPLE 13. Mapping the Transcriptional Start Site

Primer extension was carried according to the procedure of Metraux et al., PNAS,86:896-900 (1988). Briefly, 30 μg of maize pith total RNA were annealed with the primer in 50 mM Tris pH 7.5, 40 mM KCl, 3 mM MgCl2 (RT buffer) by heating to 80°C for 10 minutes and slow cooling to 42°C. The RNA/primer mix was allowed to hybridize overnight. Additional RT buffer, DTT to 6 mM, BSA to 0.1 mg/ml, RNAsin at 4 U/ml and dNTP's at 1 mM each were added. Then 8 units AMV reverse transcriptase were added and reaction placed at 37°C for one hour. The primer used was 5'-CCGTTCGTTC CTCCTTCGTC GAGG-3' (SEQ ID NO:73), which starts at +90 bp relative to the transcription start. See Fig. 29A. A sequencing ladder using the same primer as in the primer extension reaction was generated using the 4.8 Kb genomic clone to allow determination of the transcriptional start site. The sequencing reaction was carried out as described in Example 12.

RNase protection was used to determine if the the 371 bp sequence from +2 bp to +373 bp (start of cDNA) was contiguous or if it contained one or more introns. A 385 bp SphI-NcoI fragment spanning +2 bp to +387 bp relative to transcriptional start see Fig. 29B was cloned into pGEM-5Zf(+) (Promega) and transcribed using the Riboprobe Gemini system (Promega) from the SP6 promoter to generate radioactive antisense RNA probes as described by the supplier. RNase protection was carried out as described in Sambrook et al. pBR322 (cut with HpaII and end labelled with 32P-dCTP) and Klenow fragment were used molecular weight markers. Gels were 6% acrylamide/7M urea (BRL Gel-Mix 6) and were run at 60 watts constant power.

EXAMPLE 14. Genomic Southern Blots

Genomic DNA was isolated from maize line 211D using the procedure of Shure et al., supra. 8 μg of genomic DNA were used for each restriction enzyme digest. The following enzymes were used in the buffer suggested by the supplier: BamHI, EcoRI, EcoRV, HindIII, and SacI. Pith cDNA clone number 8-2 was used for estimating gene copy number. The digested

DNA was run on a 0.7% agarose gel using Tris-Borate-EDTA buffer system. The gel was pretreated with 250 mM HCl for 15 min. to facilitate transfer of high molecular weight DNA. The DNA was transferred to Nitroplus 2000 membrane and subsequently probed with the pith cDNA 8-2. The blot was washed as described in Example 10.

EXAMPLE 15. PCR Material and Methods

PCR reactions were preformed using the GeneAmp DNA Amplication reagent kit and AmpliTaq recombinant Taq DNA polmerase (Perkin Elmer Cetus). Reaction condition were as follows: 0.1 to 0.5 uM of each of the two primers used per reaction, 25 ng of the pith 4.8 Kb EcoRI fragment in Bluescript, plus the PCR reaction mix described by the supplier for a total volume of 50 uL in 0.5 mL GeneAmp reaction tube (Perkin Elmer Cetus). The DNA Thermal Cycler (Perkin Elmer Cetus) using the Step-Cycle program set to denature at 94°C for 60 s, anneal at 55°C for 60 s, and extend at 72°C for 45 s followed by a 3-s-per-cycle extension for a total of 30 cycles. The following primer sets were used: I. 83 X 84, -429 bp to -2 bp; II. 49 x 73, -69 bp to +91 bp; III. 38 X 41, +136 bp to +258 bp; and IV. 40 X 75, +239 bp to +372 bp. These are marked on Fig. 24.

EXAMPLE 16. Isolation of a Pith-Preferred Gene.

A cDNA library derived from pith mRNA cloned into Lambda Zap and screened using first strand cDNA derived from either pith or seed mRNA. Clones which hybridized with only the pith probe were plaque purified and again screened. Clones passing the second screen were used as probes in northern blots containing RNA from various maize tissues.

EXAMPLE 17. Gene Structure and Sequence Analysis.

The 1.2 Kb insert of the cDNA clone 8-2 was sequenced using the dideoxy method of Sanger et al., supra. Likewise, the genomic equivalent contained on a 4.8 Kb EcoRI fragment in Bluescript denoted as pCIB5601, was sequenced. This information revealed that the genomic copy of the coding region spans 1.7 Kb and contains five introns. The mRNA transcript represents six exons. This is shown in Fig. 24. The exons range in size from 43 bp to 313 bp and the introns vary in size from 76 bp to 130 bp. The entire sequence of the gene and its

corresponding deduced amino acid sequence are shown in Fig. 24 (SEQ ID NOS:18 and 19).

This gene encodes a protein of 346 amino acids with a molecular mass of about 38 kD. As illustrated in Table 1, the predicted protein shows 62% similarity and 41% identity with the subunit protein of *Pseudomonas aeruginosa* and has high homology with trpA proteins from other organisms.

Table 1

Conservation of TrpA sequences between a maize TrpA gene and other organisms.

Organisms compared	% amino acid Similarity	% amino acid Identity
Haloferax volancii	56.4	36.1
Methanococcus voltae	58.1	35.1
Pseudomonas aeruginosa	62.5	41.8
Neurospora crassa	61.4	39.3
Saccharomyces cerevisiae	56.7	36.1

Similarity groupings, I=L=M=V, D=E, F=Y, K=R, N=Q, S=T

Similarities and indentities were done using the GAP program from UWGCG.

Crawford et al., Ann. Rev. Microbiol., 43:567-600 (1989), incorporated herein by reference, found regions of conserved amino acids in bacterial trpA genes. These are amino acids 49 to 58, amino acids 181 to 184, and amino acids 213 to 216, with the rest of the gene showing greater variability than is seen in the TrpB sequence. An alignment of known trpA proteins with the maize TrpA protein (not shown) illustrates that the homology between the maize gene and other trpA proteins is considerable. Also, it is comparable to the level of homology observed when other TrpA proteins are compared to each other as described in Crawford et al., supra.

To determine the location of the transcription start site and whether or not there were introns present in this region, four polymerase chain reaction (PCR) generated fragments of about 122 bp to 427 bp from the region -429 bp to +372 bp were used for northern analysis. The results of the northerns showed that PCR probes II, III, IV hybridized to pith total RNA and PCR probe I did not hybridize. This indicated that the transcription start was in the -69 bp to +90 bp

region. To more precisely locate the transcriptional start site, primer extension was employed. Fig. 28A shows that when a primer (#73) located at +90 bp relative to the transcriptional start is used for primer extension, the transcriptional start site is located at +1, 1726 bp on the genomic sequence.

The first ATG from the transcriptional start site is at +114 bp. This is the ATG that would be expected to serve as the site for translational initiation. This ATG begins an open reading that runs into the open reading frame found in the cDNA clone. The first 60 amino acids of this predicted open reading frame strongly resemble a chloroplast transit peptide. See Berlyn et al. PNAS, 86:4604-4608 (1989) and Neumann-Karlin et al., EMBO J., 5:9-13 (1986). This result suggests that this protein is targeted to a plastid and is likely processed to yield the active protein. Transient expression assays in a maize mesophyll protoplast system using a maize optimized B.t. gene driven by the trpA promoter showed that when the ATG at +114 bp is used as the fusion point, the highest levels of expression are obtained. Using either of the next two ATGs in the sequence substantially reduces the level of expression of the reporter gene. The ATG at +390 bp gave some activity, but at a much lower level than the +114 ATG, and the ATG at +201 bp gave no activity.

Athough a number of TATA like boxes are located upstream of the upstream of the transcriptional start site at +1 bp, the TATAAT at -132 bp is most like the plant consensus of TATAAA. See Joshi, Nuc. Acids Res., 15:6643-6653 (1987). The presumptive CCAAT like box was found at -231 bp . The nucleotide sequence surrounding the ATG start (GCGACATGGC; see SEQ ID NO:18) has homology to other maize translation starts as described in Messing et al., Genetic Engineering of Plants: An Agricultural Perspective, Plenum Press, pp. 211-227 (1983), but differs from that considered a consensus sequence in plants (ANNATGGC). See, Joshi, above. The presumptive poly(A) addition signal is located at 3719 bp (AATAAA) on the genomic sequence, 52 bp from the end of the cDNA. The sequence matches known sequences for maize as described in Dean et al., Nuc. Acids Res., 14:2229-2240 (1986), and is located 346 bp downstream from the end of protein translation. See Dean et al., Nuc. Acids Res., 14:2229-2240 (1986). The 3' untranslated sequence of the cDNA ends at 3775 bp on the genomic sequence.

Fig. 27 shows a Southern blot of maize 211D genomic DNA with the approximate gene copy number as reconstructed using pith gene 8-2 cDNA. From the restriction digests and reconstruction there appear to be 1-2 copies of the gene present per haploid genome. There do not appear to be other genes with lower levels of homology with this gene. Therefore, this represents a unique or small member gene family in maize.

EXAMPLE 18. RNase Protection

The structure of the 5' end of the mRNA was determined using RNase protection. The RNase protection was carried out using a probe representing 385 nt from +2 bp to +387 bp. This region from the genomic clone was placed in the RNA transcription vector pGEM-5Zf(+) and a 32P labelled RNA probe generated using SP6 polymerase. The probe and the extra bases from the multiple cloning site produce a transcript of 461 nt. The probe was hybridized with total pith RNA and subsequently digested with a mixture of RNase A and T1 and the protected fragments analyzed on denaturing polyacrylamide gels. Analysis of the gels shows a protected fragment of about 355 nt and another fragment of about 160 nt. See Fig. 28B.

The fact that primer extension using a primer (#73) at +80 bp produces a product of 90 NT in length argues that the 5' end of the transcript is located at position +1 bp. Primer extension from a primer in this region produces a product, so one would expect this also to be detected by the RNase protection assay. This primer is located in the 5' region of the RNase protection probe. The cDNA clone contains sequences present in the 3' end of the RNase protection probe and hence were expected to be protected in this assay. Since only one band is present on the gel which could account for both of these sequences, we are confident that the protected fragment is indeed the larger band and that the smaller single band is an artifact. If there were an intron in this region, fragments from each end would be present in the probe, and hence would be detectable on the gel. Of the two bands seen, one of them appears to represent the entire 5' region, therefore we do not believe that there is an intron located in this region.

EXAMPLE 19. Complementation of *E. coli* TrpA Mutant with the Pith cDNA 8-2

E. coli strain CGSC strain 5531 from the E. coli Genetic Stock Center, Yale University (O.H. Smith lab strain designation, #M5004) with chromosomal markers glnA3,

TrpA9825, l-,IN(rrnD-rrnE), thi-1 as described in Mayer et al., Mol. Gen. Gentet., 137:131-142 (1975), was transformed with either the pith (TRpA) cDNA 8-2 or Bluescript plasmid (Stratagene) as described in Sambrook et al., supra. The transformants containing the TrpA cDNA 8-2 had the ability to grow without the presence of tryptophan on minimal medium whereas the transformants with the Bluescript (Stratagene) plasmid or untransformed control were not able to grow without tryptophan. The cells transformed with the maize TrpA gene grew very slowly with colonies visible after seven days growth at room temperature. All strains were grown on M9 minimal medium supplemented with 200 ug/ml glutamine, 0.01 ug/ml thiamine and with or without 20 ug/ml tryptophan. All transformants were checked for the presence of the appropriate plasmid by restriction enzyme analysis. Colonies growing in the absence of tryptophan all contained clone 8-2 containing the cDNA for the putative maize TrpA gene, as confirmed by Southern hybridization (data not shown). These results support the conclusion that this is the maize tryptophan synthase subunit A protein.

EXAMPLE 20. Gene Expression

The expression pattern of the pith-preferential gene throughout the plant was examined. Different maize genotypes were also examined for patterns of expression of this gene. The following tissues were used as the source of RNA for these studies: upper, middle, and lower pith, brace roots, ear shank, cob in genotype 5N984; upper, middle, lower pith, 10 day old leaves, 14 day old roots and pith from the entire plant in genotype 211D, and seed from genotype 211D which had been harvested at weekly intervals one to five weeks post-pollination. Lower pith is derived from, i.e. constitutes the two internodes above brace roots; middle pith is derived from the next three internodes; upper pith represents the last two internodes before the tassel in 60 and 70 day plants. Only two internodes were present in 39 day old plants and three internodes for 46 day old plants. Northern blot analysis shows that transcripts hybridizing with a probe derived from the pith cDNA accumulate rapidly in young pith and young leaf. As the age of the plant increases and one moves up the stalk, there is a significant decrease in the amount of transcript detected. See Figs. 25A-D. At no time is message from this gene detected in seed derived RNA, either total RNA or poly A+ RNA. See Fig. 26. Transcript is also detected in root, earshank, and sheath but not at the high levels detected in the pith and young leaf tissues.

See Figs. 25B, 25C. Some message is detected in brace roots, but only at a very low level. See Fig. 25D. Six maize undifferentiated callus lines were analyzed by northern blot analysis and no expression was found for this gene (data not shown) in any callus sample. The level of expression of this gene is extremely high since a very strong signal to a probe from TrpA gene 8-2 can be detected in pith and leaf as little as two hours after exposure of the blot to film (Fig. 25A). The amount of mRNA made is comparable to that derived from the maize phosphoenolpyruvate carboxylase gene disclosed in Hudspeth et al., Plant Mol. Biology, 12:579-589 (1989), another highly expressed maize gene. Hudspeth is incorporated herein by reference.

The expression pattern of this gene is not temporally constant. Expression is very high in the lower and middle pith of plants less than 60 days old and decreases rapidly near the top of the plant. As the plant reaches maturity, e.g. over 70 days old, the expression drops to nearly undetectable levels except in the lower pith and earshank. The accumulation of transcript in young leaf is nearly as high as that seen in lower pith but expression decreases rapidly and is undetectable in leaves over 40 days of age. Expression in leaf was found to be variable depending on the season when it is grown.

Examples 21-39 set forth below are directed to the isolation, characterization and expression analysis of a pollen-specific promoter according to the present invention.

Identification of pollen-specific proteins

Example 21. Maize Plant Growth

Maize plants (Zea mays Funk inbred 211D) were grown from seed in a vermiculite/sand mixture in a greenhouse under a 16 hour light/8 hour dark regime.

Example 22. Total Pollen Protein Isolation

Mature pollen was isolated from maize plants at the time of maximum pollen shed. It was sieved to remove debris, frozen in liquid nitrogen, and a 3-4 ml volume of frozen pollen was ground in a mortar and pestle with an equal volume of 75-150 μm glass beads. 40 ml of grinding buffer (2mM EDTA, 5mM DTT, 0.1% SDS, 100 mM Hepes pH 8) was added and the mixture was ground again. The glass beads and intact pollen grains were pelleted by low speed

centrifugation, and mixture was clarified by centrifugation at 10,000 g for 15 minutes. Protein was precipitated from the supernatant by addition of acetone to 90%.

Example 23. Pollen Exine Protein Isolation

Exine Protein was isolated from maize 211D shed pollen as described in Matousek and Tupy, J., Plant Physiology 119:169-178 (1985).

Example 24. Leaf Protein Isolation

Young leaves (about 60% expanded) were cut from the maize plant the midrib removed. Total protein was isolated as for pollen, except that the material was not frozen and grinding was in a Waring blender without glass beads.

Example 25. Kernel Protein Isolation

Ears with fully developed, but still moist kernels were removed from the plant and the kernels cut off with a scalpel. Total protein was isolated as for leaves.

Example 26. Gel Electrophoresis of Maize Proteins

Pollen, leaf and kernel proteins were separated on SDS polyacrylamide gels as described in Sambrook et al, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press: New York (1989). Following staining by Coomasie blue, protein bands from pollen, leaf and kernel were compared and abundant proteins of approximately 10 kD,13 kD, 20 kD, 45 kD, 55 kD and 57 kD were determined to be pollen specific.

Identification of Pollen-Specific cDNA clones

Example 27. Partial Sequence Determination of Pollen-Specific Proteins

Protein bands determined to be pollen-specific were purified by electroblotting from the polyacrylamide gel onto PVDF membrane (Matsudaira, P., J. Biol. Chem. 261:10035-10038 (1987)) or by reverse phase HPLC. N-terminal sequence of the purified proteins was determined by automated Edman egradation with an Applied Biosystems 470A gas-phase sequencer. Phenylthiohydantoin (PTH) amino acids were identified using an Applied Biosystems 120A PTH

analyzer. To obtain internal sequence, proteins were digested with endoproteinase Lys-C (Boehringer Mannheim) in 0.1 M Tris-HCl, pH 8.5, for 24 hours at room temperature using an enzyme:substrate ratio of 1:10. Resulting peptides were isolated by HPLC using an Aquapore C-8 column eluted with a linear acetonitrile/isopropanol (1:1 ratio) gradient (0 to 60%) in 0.1% TFA. Sequence of isolated Lys-C peptides was determined as above. The following sequences were determined for the 13kD pollen-specific protein:

N-terminus: TTPLTFQVGKGSKPGHLILTPNVATI (SEQ ID NO:74)
LysC 61: KPGHLILTPNVATISDVVIK (SEQ ID NO:75)
LysC 54: SGGTRIADDVIPADFK (SEQ ID NO:76)
LysC 49: EHGGDDFSFTLK (SEQ ID NO:77)
LysC 43: EGPTGTWTLDTK (SEQ ID NO:78)

Example 28. Synthesis of Oligonucleotide Probes for Pollen-Specific cDNAs

Regions of peptide sequence in the 13kD protein with low codon redundancy were selected, and suitable oligonucleotide probes for the gene encoding these regions were synthesized on an Applied Biosystems 380A synthesizer. The following oligonucleotides were synthesized:

Oligo #51 5'-AA RTC RTC ABC ACC RTG YTC-3' (SEQ ID NO:79)
Oligo # 58 5'-CC YTT NCC CAC YTG RAA-3' (SEQ ID NO:80)

where the columns of nucleotides represent bases that were incorporated randomly in equal proportions at the indicated position in the oligo. Oligo #51 encodes the amino acid sequence EHGGDDF (amino acids 1 to 7 of SEQ ID NO:77) found in peptide LysC 49, and Oligo #58 encodes the amino acid sequence FQVGKG (amino acids 6 to 11 of SEQ ID NO:74) found in peptide N-terminus. Use of these mixed oligonucleotides to screen a cDNA library for the pollen-specific gene will be described below.

Example 29. Construction of a maize pollen cDNA library

Total maize RNA from maize 211D shed pollen was isolated as described in Glisen et al, Biochemistry 13:2633-2637 (1974). Poly A+ mRNA was purified from total RNA as described in Sambrook et al. Using this mRNA, cDNA was prepared using a cDNA synthesis kit purchased from Promega, following protocols supplied with the kit. The EcoRI linkers were added to the cDNA and it was ligated into arms of the cloning vector lambda Zap, purchased from Stratagene and using the protocol supplied by the manufacturer. The ligation product was packaged in a lambda packaging extract also purchased from Stratagene, and used to infect E. *coli* BB4 cells.

Example 30. Isolation of pollen-specific cDNA clones

The maize pollen cDNA library was probed using the synthetic oligonucleotides probes specific for the 13kD protein gene, as described in Sambrook et al. Briefly, about 100,000 phage plaques of the pollen cDNA library were plated and lifted to nitrocellulose filters. The filters were probed using oligonucleotides #51 and #58 which had been 32P end-labeled using polynucleotide kinase. The probes were hybridized to the filters at low stringency (50 degrees C in 1M NaCl, 10% dextran sulfate, 0.5% SDS), washed 30 minutes at room temperature and then 30 minutes at 45 degrees C in 6X SSC, 0.1% SDS, and exposed to X-ray film to identify positive clones. Putative clones were purified through four rounds of plaque hybridization. Three classes of cDNA clones were isolated. Type I contained EcoRI fragments of 0.2 kb and 1.8 kb. Type II contained EcoRI fragments of 0.6 kb, 0.5 kb and 1.0 kb, and Type III contained an EcoRI fragment of 2.3 kb.

Example 31. Characterization of Pollen-specific cDNA clones

The EcoRI fragments of the Type II cDNA clone were subcloned into the plasmid vector pBluescript SK+, purchased from Stratagene. See Fig. 29. The 0.6 kb fragment in pBluescript was named II-.6, the 0.5 kb fragment in pBluescript was named II-.5 (later renamed pCIB3169) and the 1.0 kb fragment in pBluescript was named II-1.0 (later renamed pCIB3168). As will be described below, the 0.5 kb and 1.0 kb fragments encode the maize pollen-specific CDPK gene. RNA from anthers, pollen, leaf, root and silk was denatured with glyoxal,

electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and probed separately with the three EcoRI fragments that had been labeled with 32P by random primer extension as described in Sambrook et al, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press: New York (1989). The blots were exposed to X-ray film, and an mRNA band of approximately 1.5 kb was identified with the 0.6 kb fragment probe, while the 0.5 and 1.0 kb fragments hybridized to an approximately 2.0 kb mRNA. In all cases hybridization was only seen in the pollen RNA lane, with the exception that the 0.6 kb fragment showed a slight signal in anther mRNA. The conclusion from these data was that the original cDNA clone was a fusion cDNA molecules derived from two different mRNAs. The 0.6 kb fragment was a partial cDNA of a 1.5 kb pollen-specific mRNA, and this mRNA encodes the peptides LysC 49 and N-terminus. The 1.0 and 0.5 kb fragments comprise a partial cDNA of a 2.0 kb pollen-specific mRNA unrelated to the peptides and oligonucleotide probes used for probes. This conclusion was verified when the fragments were sequenced using the dideoxy chain termination method as described in Sambrook et al. The cDNA sequence is shown in Fig. 30 (SEQ ID NO:20).

Example 32. Determination of specificity of mRNA expression

To determine if the 2.0 kb RNA represented by cDNA clones pCIB3169 and pCIB3168 were present only in pollen, total RNA was isolated from maize 211D roots, leaves, pollen, anthers or silks. The RNAs were denatured with glyoxal, electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and probed with 32P-labeled EcoRI insert from plasmid pCIB3168 or pCIB3169, all using standard techniques as described in Sambrook et al, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press: New York (1989). Exposure of this blot to photographic film demonstrates that the gene represented by these two clones is only transcriptionally active in the pollen (Fig. 31).

Identification of a Pollen-Specific Promoter

Example 33. Construction of a Maize Genomic DNA Library

Genomic DNA from maize line 211D young shoots was isolated as described in Shure et l, Cell 35:225-233 (1983). The DNA was provided to Stratagene, where a genomic DNA

library was constructed by cloning Sau3AI partially digested DNA into Stratagene's Lambda Dash cloning vector.

Example 34. Genomic DNA Blot Hybridization to Determine Gene Copy Number.

Genomic DNA from maize line 211D was digested with a number of restriction enzymes, the individual digests electrophoresed on an agarose gel, transferred to nitrocellulose, and probed with 32P-labeled EcoRI insert from plasmid pCIB3168 (1.0 kb fragment), pCIB3169 (0.5 kb fragment) or clone II-.6 using standard techniques described in Sambrook et al. More than 10 bands were detected by the II-.6 probe on most digests, indicating that this cDNA is derived from a large, multigene family. Probing with the 1.0 kb fragment detected from 3 to 6 bands, and probing with the 0.5 kb fragment detected only from 1 to 3 bands which were a subset of those detected by the 1.0 kb fragment. Due to the smaller gene family size detected by the 1.0 kb and 0.5 kb fragments, it was decided to attempt to isolate the genomic clone corresponding to them.

Example 35. Isolation of a pollen-specific genomic clone

The Stratagene maize 211D genomic library was screened by probing plaque lifts with 32P labeled inserts from plasmid pCIB3168 (1.0 kb fragment) and pCIB3169 (0.5 kb fragment) using standard procedures as described in the Stratagene manual accompanying the library. Using this strategy, Lambda clone MG14 was isolated, and it hybridized to both probes. The 9.0 kb BamHI fragment of MG14, which also hybridized to both probes, was subcloned into the BamHI site of pBluescript SK+ to create plasmid pCIB379. 1800 bp of pCIB379, in the region corresponding to the cDNA sequence, was sequenced as described above. Comparison of the cDNA and genomic sequences showed only 91% identity. pCIB379 insert represents a related pollen-specific gene.

A second maize 211D genomic library was constructed in the vector lambda GEM-11, purchased from Promega, using the procedures described in the Promega manual. Screening this un-amplified library as above yielded clone GEM11-1, which hybridized to both 0.5 and 1.0 kb probes. The 20kb HindIII fragment of GEM11-1, which also hybridized to both probes, was subcloned into the HindIII site of pBluescript SK+ to yield pCIB3166. The DNA sequence of

4.1kb of pCIB3166 was determined (Fig. 35; SEQ ID NO:26) and after accounting for six introns in the genomic clone, was 100% identical to the cDNA sequence of pCIB3168 and pCIB3169. Comparison of the pCIB3166 sequence to the Genbank/EMBL database revealed that the 5' portion, through the 3 exon, was 34.6% identical to rat calmodulin-dependent protein kinase II at the amino acid level (Fig. 32), while the fourth through seventh exons were 39.4% identical to human calmodulin. See Fig. 33. No other pollen-specific kinase has been described, and at the time this a protein combining kinase and calmodulin domains was unknown. Subsequently, Harper et al., Science 252:951-954 (1991) have disclosed the cDNA sequence of a similar protein from soybean, although this gene is not pollen-specific in expression. Comparison of the soybean calcium-Dependent Protein Kinase (CDPK) and the maize pollen CDPK reveals 38% identity at the amino acid level. See Fig. 34.

Example 36. Identification of the Promoter's Transcriptional Start Site by Primer Extension

Oligonucleotide PE51, with the following sequence was synthesized as a primer.

5'-TGGCCCATGGCTGCGGCGGGGAACGAGTGCGGC-3' (SEQ ID NO:81)

Primer extension analysis was carried out on polyA+ pollen mRNA as described in Metraux et al., PNAS USA 86:896-890 (1989). The transcription initiation site was determined to be between bases 1415 and 1425 on the partial sequence of pCIB3166 shown in Fig. 35.

Testing Promoter Function in Transgenic Plants

Example 37. Construction of promoter vectors for plant transformation

To demonstrate that the pollen CDPK promoter can drive expression of a linked gene in transgenic plants, a gene fusion of the pollen CDPK promoter to the Beta-glucuronidase gene of *E. coli* was constructed as follows. The 10kb BamHI fragment from lambda GEM11-1 containing the first exon and part of the first intron of the pollen CDPK gene plus 9 kb upstream of the gene was subcloned into the BamHI site of pBluescript SK+ to create plasmid pCIB3167. The 2.3 kb BamHI-HindIII fragment from pCIB3167 was subcloned into the BamHI and HindIII sites of pBluescript SK+ to create plasmid pSK105. The pSK105 was digested with AvaI and HindIII, and the1.75 kb HindIII-AvaI fragment was isolated on an agarose gel. A PCR reaction

was run under standard conditions as described in Sambrook et al. using intact pSK105 as a template and the following primers:

#42: 5'-AGCGGTCGACCTGCAGGCATGCGATCTGCACCTCCCGCCG-3' (SEQ ID NO:82)

#43: 5'-ATGGGCAAGGAGCTCGGG-3 (SEQ ID NO:83)

The PCR reaction products were digested with AvaI and SalI and the resulting fragment isolated on an agarose gel. pBluescript SK+ was digested with HindIII and SalI. The 1.75 kb HindIII-AvaI fragment, PCR derived AvaI-SalI fragment, and pBluescript vector with HindIII and SalI ends were ligated in a three way ligation to create plasmid pSK110.

A fusion of the promoter fragment in pSK110 to the Beta-glucuronidase (GUS) gene was created by digesting pSK110 with HindIII and SaII, isolating the 1.9kb fragment on an agarose gel and ligating it into HindIII and SaII sites of pCIB3054, to create plasmid pKL2, a plasmid derived from pUC19 containing the GUS gene followed by plant intron from the maize PEPC gene and a polyA signal from cauliflower mosaic virus. This promoter fusion was inactive in plants, probably due to the presence of out of frame ATG codons in the leader sequence preceding the GUS gene ATG.

A function fusion of the promoter was created by digesting pKL2 with XbaI and SalI to remove the previous fusion junction. A new fusion junction was produced in a PCR reaction using pSK105 as a template and the following primers:

#SK50: 5'-CCCTTCAAAATCTAGAAACCT-3' (SEQ ID NO:84)

#SK49: 5'-TAATGTCGACGAACGGCGAGAGATGGA-3' (SEQ ID NO:85)

The PCR product was digested with XbaI and SalI and purified on an agarose gel. The purified fragment was ligated into the XbaI and SalI sites of pKL2 to created plasmid pCIB3171. This plasmid contains a functional fusion of pollen CDPK promoter and GUS which directs expression the GUS gene exclusively in pollen.

To create a vector containing the pollen CDPK promoter-GUS fusion suitable for use in *Agrobacterium tumefaciens*-mediated plant transformation, the fusion gene was isolated from pCIB3171 by digestion with HindIII and SalI. The resulting fragment was ligated into the HindIII and SalI sites of pBI101 (purchased from Clontech) to create plasmid pCIB3175.

Example 38. Production of Transgenic Plants

pCIB3175 was transformed into Agrobacterium tumefaciens containing the helper plasmid pCIB542, and the resulting culture used to transform leaf disks from tobacco shoot tip cultures as described by Horsch et al., Science 227:1229-1231 (1985) except that nurse cultures were omitted and selection was on 100 mg/l kanamycin. Transgenic plants were regenerated and verified for presence of the transgene by PCR.

Example 39. GUS Gene Expression Analysis

Pollen from primary transformants and their progeny were analyzed histochemically for expression of the GUS gene as described by Guerrero et al., Mol. Gen. Genet. 224:161-168 (1990). The percentage of pollen grains expressing the GUS gene, as demonstrated by blue staining in the X-gluc buffer, is shown in the table below.

Plant Number	% Blue Pollen
PP1-51	28%
PP1-54	54%
PP1-55	none
PP1-61	very few
PP1-63	51%
PP1-67	15%
PP1-80	10%
PP1-83	12%

Primary transformants in which a single pollen CDPK promoter-GUS gene was integrated would produce a maximum 50% GUS positive pollen due to segregation of the single gene. Flouometric GUS assays were done on pollen, stem, root, leaf and pistil tissue of selected plants to demonstrate the specificity of pollen CDPK promoter expression. Assays were performed as described in Jefferson, Plant Mol. Biol. 14:995-1006 (1990), and GUS activity values are expressed as nmoles MU/ug protein/minute.

Plant number	Tissue	GUS Activity	Untransformed Plant GUS Activity	Net GUS Activity
PP1-51	stem	0.01	0.02	0
	leaf	0	0	0
	root	0.15	0.10	0.05
	pistil	0.02	0.01	0.01
	pollen	0.24	0.02	0.22
PP1-54	stem	0.01	0.02	0
	leaf	0	0	0
	root	0.13	0.1	0.03
	pistil	0.01	0.01	0
	pollen	0.60	0.02	0.58
PP1-63	stem	0.01	0.02	0
	leaf	0	0	0
	root	0.07	0.1	0
	pistil	0.01	0.01	0
	pollen	0.57	0.02	0.55

Examples 40-50 are directed primarily to the preparation of chimeric constructs, i.e. recombinant DNA molecules, containing constitutive, tissue-preferred, or tissue-specific promoters operably linked to an instant B.t. gene, insertion of same into vectors, production of transgenic platns containing the vectors, and analysis of expression levels of B.t. proteins of the transgenic plants.

EXAMPLE 40: Construction Of Maize Optimized Bt Transformation Vectors

To demonstrate the effectiveness of the synthetic Bt cryIA(b) gene in maize, the PepC and pith specific promoters are fused to the synthetic Bt cryIA(b) gene using PCR. Oligomers designed for the PCR fusions were:

(PEPC)

KE99A28 = 5'-TGCGGTTACC GCCGATCACATG-3' (SEQ ID NO:86)

KE97A28 = 5'-GCGGTACCGC GTCGACGCGG ATCCCGCGGC GGGAAGCTAAG-3' (SEQ ID NO:87)

(PITH)

KE100A28 = 5'-GTCGTCGACC GCAACA-3' (SEQ ID NO:88)

KE98A28 = 5'-GCGGTACCGC GTTAACGCGG ATCCTGTCCG ACACCGGAC-3' (SEQ ID NO:89)

KE104A28 = 5'-GATGTCGTCG ACCGCAACAC-3' (SEQ ID NO:90)

KE103A28 = 5'-GCGGTACCGC GGATCCTGTC CGACACCGGA CGGCT-3' (SEQ ID NO:91)

PCR primers are designed to replace the Nco I sites in the 5' untranslated leader region of each of these tissue specific genes (containing ATG translational start sites) with Bam HI sites to facilitate cloning of the synthetic cryIA(b) gene into this Bam HI site. Subsequent construction of vectors containing the tissue specific promoters fused to the synthetic cryIA(b) gene and also containing the 35S:PAT:35S marker gene involves several intermediate constructs.

1. pCIB4406 (35S:synthetic-cryIA(b):pepC ivs#9:35S)

pCIB4406 contains the 2 Kb Bam HI\Cla I synthetic cryIA(b) gene fused with the CaMV 35S promoter (Rothstein et al., Gene 53:153-161 (1987)). The gene also contains intron #6 derived from the maize PEP carboxylase gene (ivs#9) in the 3' untranslated region of the gene, which uses the CaMV 3' end. (PNAS USA, 83:2884-2888 (1986), Hudspeth et al., Plant Molecular Biology, 12: 579-589 (1989)). pCIB4406 is ligated and transformed into the "SURE" strain of *E. coli* cells (Stratagene, La Jolla, CA) as described above. One mutation is found in pCIB4406's cryIA(b) gene at amino acid #436 which resulted in the desired Phe being changed to a Leu. pCIB4406 is fully active against European corn borer when tested in insect bioassays and produces a CryIA(b) protein of the expected size as determined by western blot analysis.

2. pCIB4407 (35S:synthetic-cryIA(b):pepC ivs#9:35S +
35S:PAT:35S)

pCIB4407 is made from an approximately 4 Kb Hind III\Eco RI fragment containing the 35S:PAT:35S gene, and the 3.1 Kb\Hind III\Eco RI 35S:synthetic-cryIA(b):35S gene from pCIB4406. pCIB4407 is ligated and transformed into "SURE", DH5alpha, and HB101 strains of *E. coli* using standard procedures (Sambrook et al.). The synthetic cryIA(b) gene has the same properties as its precursor pCIB4406.

3. pCIB4416 (35S:synthetic-cryIA(b):pepC ivs#9:35S +

35S:PAT:35S + 35S:Adh intron:GUS:35S.)

pCIB4407 is cut with Eco RI and treated with calf intestinal alkaline phosphatase (CIP) under standard conditions (Sambrook et al.) to produce an about 7.2 Kb fragment that is ligated with a 3.4 Kb Eco RI 35S:Adh\GUS:35S fragment to produce pCIB4416. Ligations and transformations into "SURE" cells is as described above. The synthetic cryIA(b) gene in pCIB4416 has the same properties as the gene in pCIB4406.

4. pCIB4418 (35S:synthetic-cryIA(b):pepC ivs#9:35S)

pCIB4406 is digested with Apa I and Bam HI and treated with CIP. pCIB4406 is digested with Bam HI and Nsp I. pBS123#13 is digested with Nsp I and Apa I. A three-way ligation is made consisting of a 4.3 Kb Apa I\Bam HI fragment from pCIB4406, a 1.3 Kb Bam HI\Nsp I fragment from pCIB4406, and a 170 bp Nsp I\Apa I fragment from pBS123#13 to form pCIB4418. The host *E. coli* strain for pCIB4418 is HB101.

5. pCIB4419 (35S:synthetic-cryIA(b):pepC ivs#9:35S +

35S:PAT:35S + 35S:Adh intron:GUS:35S.)

pCIB4416 and pCIB4418 are digested with Bst E II and Eco NI and fragments of pCIB4416 are treated with CIP. A 9.1 Kb fragment from pCIB4416 ligated to a 1.4 Kb fragment from pCIB4418 to form pCIB4419. pCIB4419 transformed in HB101 competent *E. coli* cells demonstrates full activity in insect bioassays against European corn borer.

6. pCIB4420 (Pith:synthetic-cryIA(b):PEPC ivs#9:35S +

35S:PAT:35S)

Intermediate constructs in making pCIB4420 are pBTin1, pBtin2, p4420A and pBtin3. pBtin1 (pith promoter:second half of the synthetic Bt gene + 35S:PAT:35S) is made by ligating the 2.1 Kb Xba I\Nco I pith promoter fragment from plasmid pith(3-1) with a 5.2 Kb Xba I\Nco I fragment from pCIB4407. pBtin2 is an intermediate construct containing the pith promoter modified with a 210 bp PCR fragment made using primers KE100A28 and KE98A28 listed above. The PCR reaction mix contains approximately 100 ng of a 2.1 Kb Bam HI\Nco I pith

promoter fragment with 100 pmol of each oligomer, 200 nM of each dNTP, 1 X buffer (Cetus) and 2.5 units of thermal stable polymerase. Since the Tm is relatively low (between 40° and 50°C), PCR reactions are run with the following parameters:

denaturation cycle: 94°C for 1 minute

annealing cycle : 37°C for 1 minute

extension cycle : 72°C for 45 seconds (+ 3 seconds per cycle)

number of cycles: 25

PCR reactions are treated with proteinase K as described above prior to cutting with Sal I\Kpn I followed by phenol\chloroform extraction and ethanol precipitation as described above. The 210 bp fragment is purified on a 2 % Nusieve gel and extracted from the gel using Millipore's filter units. The 210 bp Sal I\Kpn I fragment is ligated to the 4.9 Kb Sal I\Kpn I fragment from pith(3-1) to make pBtin2. p4420A (pith:synthetic-Bt:Pep intron:35S + 35S:PAT:35S) is made with a three-way ligation consisting of a 700 bp Nsi I\Bam HI fragment from pBtin2, a 1.8 Kb Bam HI\Bst E II fragment from pCIB4418, and a 5.9 Kb Bst E II\Nsi I fragment from pBtin1. After p4420A is made three mutations are discovered in pBtin2. A second PCR fragment is made to modify the Nco I site in the pith leader using primers KE104A28 and KE103A28 with Tm values around 65°C. The PCR reaction mix is identical to that listed above with the addition of glycerol to 20% to reduce mutations in G+C rich areas (Henry et al., Plant Molecular Biology Reporter 9(2):139-144, 1991). PCR parameters are as follows:

File I: 94°C : 3 minutes , 1 cycle

File II: 60°C : 1 minute

94°C : 1 minute

25 cycles

File III: 72°C : 5 minutes, 1 cycle

PCR reactions are treated as above and cut with restriction endonucleases Sal I and Kpn I. The 210 bp Sal I\Kpn I PCR (glycerol in the reaction) fragment is ligated to the 4.9 Kb Sal I\Kpn I fragment from plasmid pith(3-1) to make pBtin3. Sequence data on pBtin3-G#1 shows this PCR generated fragment to be correct.

pBtin3-G#1 is used to make pCIB4420 (also called p4420B "G#6"). pCIB4420 is constructed with a three-way ligation using the 700 bp Nsi I\Bam HI fragment from pBtin3-G#1, a 1.8 Kb Bam HI\Bst E II fragment from pCIB4418, and a 5.9 Kb Bst E II\Nsi I fragment from pBtin1. pCIB4420 is used in mesophyll protoplast experiments and demonstrates full activity of the synthetic cryIA(b) gene against European corn borer.

7. pCIB4413 (PEPC:synthetic-Bt (Phe mutation):PEPC intron:35S.)

A fusion fragment is generated by PCR using primers KE99A28 and KE97A28 with a 2.3 KB Hind III\Sal I template from pGUS4.5. The PCR mix contains the same concentration of primers, template, dNTPs, salts, and thermal stable polymerase as described above. PCR reaction parameters are:

denaturation cycle : 94°C for 1 minute

annealing cycle : 55°C for 1 minute

extension cycle : 72°C for 45 seconds (+ 3 seconds per cycle)

number of cycles: 30

After completion, PCR reactions are treated with proteinase K followed by phenol\chloroform extraction and ethanol precipitation as described above prior to cutting with restriction endonucleases Bam HI and Bst E II.

pCIB4413 is made with a three-way ligation using the 210 bp Bam HI\Bst E II PCR fragment, a 4.7 Kb Bam HI\Hind III fragment from pCIB4406, and a 2.2 Kb Hind III\Bst E II fragment from pGUS4.5.

8. pCIB4421 (PEPC:synthetic-cryIA(b):PEPC intron:35S.)

pCIB4421 is made to replace the synthetic cryIA(b) gene containing the Phe mutation in pCIB4413 with the synthetic cryIA(b) gene from pCIB4419. pCIB4421 is made by ligating a 5.2 Kb Bam HI\Sac I fragment from pCIB4413 with a 1.9 Kb Bam HI\Sac I fragment from pCIB4419.

9. pCIB4423 (PEPC:synthetic-cryIA(b):PepC intron:35S + 35S:PAT:35S)

The 2.4 Kb Bam HI\Hind III PEPC promoter fragment from pCIB4421 is ligated to the 6.2 Kb Bam HI\Hind III fragment in pCIB4420 to make pCIB4423. The Hind III site is deleted by exonucleases in the cloning of pCIB4423. pCIB4423 contains the synthetic cryIA(b) gene under the control of the PEPC promoter, and the PAT gene under the control of the 35S promoter.

10. Synthetic cryIA(b) gene in *Agrobacterium* strains:

Agrobacterium strains made with the synthetic cryIA(b) gene allow transfer of this gene in a range of dicotyledenous plants. *Agrobacterium* vector pCIB4417 contains the 3.3 Kb Hind III\Eco RI 35S:synthetic-CryIA(b):PepC:ivs#9:35S fragment from pCIB4406 (Phe mutation) ligated to the 14 Kb Hind III\Eco RI fragment from pBI101 (Clontech). Using electroporation, pCIB4417 is transferred into the *A. tumefaciens* strain LBA4404 (Diethard et al., Nucleic Acids Research, Vol17:#16:6747, 1989.).

200 ng of pCIB4417 and 40 ul of thawed on ice LBA4404 competent cell are electroporated in a pre-cooled 0.2 cm electroporation cuvette (Bio-Rad Laboratories Ltd.). Using Gene Pulser-TM with the Pulse Controller unit (Bio-Rad), an electric pulse is applied immediately with the voltage set at 2.5 kV, and the capacity set at 25 uF. After the pulse, cells are immediately transferred to 1 ml of YEB medium and shaken at 27 C for 3 hours before plating 10 ul on ABmin:Km50 plates. After incubating at 28 C for approximately 60 hours colonies are selected for miniscreen preparation to do restriction enzyme analysis. The final *Agrobacterium* strain is called pCIB4417:LBA4404.

EXAMPLE 41: ELISA Analysis Of Transformed Maize Protoplasts

The presence of the cryIA(b) toxin protein is detected by utilizing enzyme-linked immunosorbent assay (ELISA). ELISAS are very sensitive, specific assays for antigenic material. ELISA assays are useful to determine the expression of polypeptide gene products. Antiserum for these assays is produced in response to immunizing rabbits with gradient-purified Bt crystals (Ang et al., Applied Environ. Microbiol., 36:625-626 (1978)) solubilized with sodium dodecyl sulfate. ELISA analysis of extracts from transiently transformed maize cells is carried out using standard procedures (see for example Harlow, E., and Lane, D. in "Antibodies: A

Laboratory Manual", Cold Spring Harbor Laboratory Press, 1988). ELISA techniques are further described in Clark et al., Methods in Enzymology, 118:742-766 (1986); and Bradford, Anal. Biochem., 72:248 (1976). Thus, these procedures are well-known to those skilled in the art. The disclosure of these references is hereby incorporated herein by reference.

ELISA assays are performed to detect the production of CryIA(b) protein in maize protoplasts. Protein produced is reported below as ng of Bt per mg total protein (ng Bt/mg). Each construct was tested twice.

pCIB3069 No detectable Bt (both tests)

pCIB4407 21,900 ng Bt/mg total protein,

21,000 ng Bt/mg total protein

The transformed maize cells produce high levels, on the order of approximately 20,000 ng of Bt CryIA(b) protein per mg total soluble protein, of the Bt IP when transformed with the maize optimized Bt gene. The level of detection of these ELISA based assays is about 1 to 5 ng CryIA(b) protein per mg protein. Therefore, the maize optimized Bt gene produces as much as approximately a 20,000 fold increase in expression of this protein in maize cells.

EXAMPLE 42: Assay Of Extract From Transformed Protoplasts For Insecticidal Activity Against European Corn Borer

Western blot analysis is also performed using extracts obtained from maize cells which had been transiently transformed with DNA to express the maize optimized gene. When examined by western blots, this protein appears identical with the protein produced in *E. coli*. In contrast, as demonstrated in Example 6 above, no detectable Bt cryIA(b) insecticidal protein is produced by maize cells transformed with comparable vectors attempting to express the native Bt derived coding region.

Qualitative insect toxicity testing can be carried out using harvested protoplasts. Suspensions are prepared for each replicate tested in all bioassays. A replicate is considered positive if it causes significantly higher mortality than the controls. For example, replicates are tested for their activity against insects in the order *Lepidoptera* by using the European corn borer, *Ostrinia nubilalis*. One-hundred μl of a protoplast suspension in 0.1% Triton X-100 is pipetted onto the surface of artificial Black cutworm diet, (Bioserv, Inc., Frenchtown, NJ; F9240) in 50

mm X 10 mm snap-cap petri dishes. After air drying 10 neonatal larvae are added to each plate. Mortality is recorded after about 4 days. When this protein is fed to European corn borers, it produces 100% mortality.

EXAMPLE 43: Expression Of Synthetic Bt In Maize Mesophyll Protoplasts

The general procedure for the isolation of corn mesophyll protoplasts is adapted from Sheen et al., The Plant Cell, 2:1027-1038 (1990). The protoplast transformation system used in Sheen et al. is modified by using PEG mediated transformation, rather than electroporation. That procedure, as well as changes made in the isolation procedure, is described below.

Maize Mesophyll Protoplast Isolation/Transformation

1. Sterilize and germinate corn seeds for leaf material. Seedlings are grown in the light at 25C.

2. Surface sterilize leaf pieces of 10-12 day old seedlings with 5% Clorox for 5 minutes followed by several washes with sterile distilled water.

3. Aliquot enzyme solution (see recipe below); 25 ml/dish (100x25 mm petri dish).

4. Remove any excess water from leaves and place 6-8 2 inch pieces in each dish of enzyme. 14 plates are usually set up with the leaf material from about 100 seedlings.

5. Cut leaves in longitudinal strips as thin as possible (2-5 mm).

6. Shake slowly at 25C for 6.5 to 7 hours. Cover plates so that incubation takes place in the dark.

7. Before filtering protoplasts, wash 100 um sieves with 10 ml 0.6 M mannitol. Pipet protoplasts slowly through sieves. Wash plates with 0.6 M mannitol to gather any protoplasts left in the dishes.

8. Pipet filtered liquid carefully into 50 ml sterile tubes. Add equal volumes of 0.6 M mannitol to dilute.

9. Spin for 10 minutes at 1000 rpm/500 g in table-top centrifuge (Beckman Model TJ-6).

10.Remove enzyme solution and discard. Resuspend pellets carefully in 5 ml mannitol. Pool several pellets. Bring volume to 50 ml with 0.6 M mannitol and spin.

11. Resuspend to a known volume (50 ml) and count.

12. After counting and pelleting, resuspend protoplasts at 2 million/ml in resuspending buffer (recipe below). Allow ppts to incubate in the resuspending buffer for at least 30 min before transformation.

Transformation:

1. Aliquot plasmids to tubes (Fisherbrand polystyrene 17 x 100 mm Snap Cap culture tubes); at least three replicates per treatment; use equimolar amounts of plasmids so that equal gene copy numbers are compared.

2. Add 0.5 ml protoplasts and 0.5 ml 40% PEG made with 0.6 M mannitol.

3. Shake gently to mix and incubate at 25C for 30 min.

4. Add protoplast culture media at 5 min intervals: 1,2,5 ml

5. Spin for 10 min at 1000 rpm/500 g.

6. Remove liquid from pellet and resuspend in 1 ml culture media (BMV media)

7. Incubate overnight at 25C in the dark.

Recipes:

Enzyme Solution

0.6 M mannitol

10 mM MES, pH 5.7

1 mM $CaCL_2$

1 mM $MgCl_2$

0.1% BSA

filter-sterilize

To this solution, add the following enzymes:

1% Cellulase RS, and 0.1% Macerozyme R10

Wash Buffer: 0.6 M mannitol, filter-sterilize

Resuspending Buffer: 0.6 M mannitol, 20 mM KCl, filter-sterilize

Culture Media: BMV media recipe from:

Okuno et al., Phytopathology 67:610-615 (1977).

0.6 M mannitol

4 mM MES, pH 5.7

0.2 mM KH_2PO_4

1 mM KNO_3

1 mM $MgSO_4$

10 mM $CaCl_2$

1X K3 micronutrients

filter-sterilize

ELISA analysis of transformed protoplasts is done one day after transformation. ELISA's are done as previously described. The following three experiments are done with maize inbred line 2T1D. Of course, other lines of maize may be used. 50 ug of plasmid pCIB4419 and equimolar amounts of other plasmids are used. Total soluble protein is determined using the BioRad protein assay. (Bradford, Anal.Biochem, 72:248 (1976).

Transformation Experiment:

Constructs tested:

1. pCIB4419 (Construct contains synthetic Bt under control of CaMV 35S promoter and 35S/PAT and 35S/GUS marker genes)
2. pCIB4420 (Construct contains synthetic Bt under control of Pith promoter and PAT marker gene)
3. pCIB4421 (Construct contains synthetic Bt under control of PEPC promoter)
4. pCIB4423 (Construct contains synthetic Bt under control of PEPC promoter and PAT marker gene) (PEPC:synthetic-cryIA(b):PepC intron:35S + 35S:PAT:35S)

In the following experiments, 10 or 11 day old 211D seedlings are analyzed for production of the Bt CryIA(b) protein in the Biorad protein assay:

Experiment 1 (11 day seedlings):

pCIB4419 15,000 ± 3,000 ng Bt/mg protein

pCIB4420	280 ± 65 ng Bt/mg protein
pCIB4421	9,000 ± 800 ng Bt/mg protein

Experiment 2 (10 day seedlings):

pCIB4419	5,000 ± 270 ng Bt/mg protein
pCIB4420	80 ± 14 ng Bt/mg protein
pCIB4421	1,600 ± 220 ng Bt/mg protein

Experiment 3 (11 day seedlings):

pCIB4419	21,500 ± 1,800 ng Bt/mg protein
pCIB4420	260 ± 50 ng Bt/mg protein
pCIB4421	11,900 ± 4,000 ng Bt/mg protein
pCIB4423	7,200 ± 3,400 ng Bt/mg protein

The above experiments confirm that both the CaMV 35S and PEPC promoters express the synthetic Bt CryIA(b) protein at very high levels. The pith promoter, while less efficient, is also effective for the expression of synthetic CryIA(b) protein.

EXAMPLE 44: Stable Expression Of Synthetic Bt In Lettuce

The synthetic Bt gene in the *Agrobacterium* vector pCIB4417 is transformed into *Lactuca sativa* cv. Redprize (lettuce). The transformation procedure used is described in Enomoto et al., Plant Cell Reports, 9:6-9 (1990).

Transformation procedure:

Lettuce seeds are suface sterilized in 5% Clorox for 5 minutes followed by several washes in sterile distilled water. Surface-sterilized seeds are plated on half strength MS media (Murashige and Skoog, Physiol. Plant. 15:473-497 (1962)).

Cotyledons of 6-day-old Redprize seedlings, grown under illumination of 3,000 lx 16 hr at 25C, are used as the explants for *Agrobacterium* infection. The base and tip of each cotyledon are removed with a scalpel. The explants are soaked for 10 minutes in the bacterial solution which have been cultured for 48 hours in AB minimal media with the apropriate antibiotics at 28C.

After blotting excess bacterial solution on sterile filter paper, the explants are plated on MS media (0.1 mg/l BA and 0.1 mg/l NAA) for 2 days. Explants are then transferred to selective media containing 500 mg/l carbenicillin and 50 mg/l kanamycin. The explants are subcultured to fresh media weekly. The growth chamber conditions are 16 hour 2,000 lx light at 25C. After approximately 4 weeks, an ELISA is done on healthy looking callus from each of four plates being subcultured. The ELISA procedure is the same as described above for protoplasts; soluble protein is again determined by the Biorad assay described above.

Results:

pCIB3021 (kan control)	0
pCIB4417 (plate 1)	0
pCIB4417 (plate 2)	505 ng Bt/mg protein
pCIB4417 (plate 3)	45 ng Bt/mg protein
pCIB4417 (plate 4)	1,200 ng Bt/mg protein

This example demonstrates that dicot plants can also show increased expression of the optimized insecticidal gene.

EXAMPLE 45. Construction of pCIB4429.

pCIB4429 contains a preferred maize pollen-specific promoter fused with the maize optimized cryIA(b) gene. The pollen-specific maize promoter used in this construct was obtained from the plasmid pKL2, described in Example 37. The maize optimized cryIA(b) gene was obtained from plasmid pCIB4418, also described in Example 37.

pKL2 is a plasmid that contains a preferred maize pollen-specific promoter fused with the *E. coli* beta-glucuronidase gene. It was constructed from plasmids pSK110 and pCIB3054. pSK110 contains the pollen specific maize promoter. pCIB3054, a pUC19 derivative, contains the *E. coli* beta-glucuronidase (GUS) gene fused with the cauliflower mosaic virus (CaMV) 35S promoter. It's construction is described elsewhere in this application. This promoter can be removed from this plasmid by cutting with SalI/HindIII to yield a fragment containing the GUS gene, a bacterial ampicillin resistance gene and a ColEI origin of replication. A second fragment contains the CaMV 35S promoter.

pCIB3054 was cut with the restriction enzymes SalI and HindIII, using standard conditions, for 2 hours at room temperature. The reaction was then extracted with phenol/chloroform using standard conditions and the DNA recovered by ethanol precipitation using standard conditions. The recovered DNA was resuspended in buffer appropriate for reaction with calf intestinal alkaline phosphatase (CIP) and reacted with 2.5 units of CIP at 37°C overnight. After the CIP reaction, the DNA was purified on an agarose gel using standard conditions described elsewhere in this application. pSK110 was cut with SalI/HindIII under standard conditions for 2 hours at room temperature and the DNA subsequently purified on an agarose gel using standard conditions. The recovered DNA fragments were ligated using standard conditions for two hours at room temperature and subsequently transformed into competent *E. coli* strain HB101 cells using standard conditions. Transformants were selected on L-agar containing 100 μg ampicillin/ml. Transformants were characterized for the desired plasmid construct using standard plasmid mini-screen procedures. The correct construct was named pKL2.

To make pCIB4429, a three way ligation was performed using standard conditions known to those in the art. The three fragments ligated were:

1) a HindIII/BamHI fragment from pCIB4418, of about 4.7 kb in size, containing the cryIA(b) gene, the bacterial ampicillin resistance gene, and the ColEI origin of replication

2) a HindIII/XbaI fragment from pKL2 of about 1.3 kb in size and containing the pollen specific promoter from maize

3) a PCR generated fragment derived from the pollen promoter with a BamHI site introduced downstream from the start of transcription. This fragment is approximately 120 bp and has ends cut with the restriction enzymes XbaI/BamHI.

The PCR fragment was generated using a 100 μl reaction volume and standard conditions described above. The primers used were:

SK50: 5'-CCC TTC AAA ATC TAG AAA CCT-3' (SEQ ID NO:84)

KE127: 5'-GCG GAT CCG GCT GCG GCG GGG AAC GA-3' (SEQ ID NO:92)

The above primers were mixed in a PCR reaction with plasmid pSK105, a plasmid that contains the pollen specific promoter from maize.

After the PCR reaction was complete, 10 μl of the reaction was run on an agarose gel. using standard condition, to make sure the reaction produced the expected size product. The remaining 90 μl was treated with proteinase K at a final concentration of 50 μg/ml for 30 min. at 37°C. The reaction was then heated at 65°C for 10 min., then phenol/chloroform extracted using standard procedures. The DNA was recovered from the supernatant by precipitating with two volumes of ethanol using standard conditions. After precipitation, the DNA was recovered by centrifuging in a microfuge. The pellet was rinsed one time with 70% ethanol (as is standard in the art), briefly dried to remove all ethanol, and the pellet resuspended in 17 μl TE buffer. 2 μl of 10X restriction enzyme buffer was added as were 0.5 μl BamHI and 0.5 μl XbaI. The DNA was digested for 1 hour at 37°C to produce a DNA fragment cut with XbaI/BamHI. After digestion with the restriction enzymes, this fragment was purified on an agarose gel composed of 2% NuSieve (FMC)/1% agarose gel. Millipore filter units were used to elute the DNA from the agarose using the manufacturer's specifications. After elution, the DNA was used in the three-way ligation described above.

After ligation, the DNA was transformed into competent *E. coli* strain HB101 cells using standard techniques. Transformants were selected on L-agar plates containing ampicillin at 100 μg/ml. Colonies that grew under selective conditions were characterized for plasmid inserts using techniques standard in the art.

EXAMPLE 46. Construction of pCIB4431, a vector for tissue specific expression of the synthetic cryIA(b) gene in plants.

pCIB4431 is a vector designed to transform maize. It contains two chimeric Bt endotoxin genes expressible in maize. These genes are the PEP carboxylase promoter/synthetic-cryIA(b) and a pollen promoter/synthetic-cryIA(b). The PEP carboxylase/cryIA(b) gene in this vector is derived from pCIB4421 described above. The pollen promoter is also described above. Fig. 20 is a map of plasmid pCIB4431. pCIB4431 was constructed via a three part ligation using the about 3.5 Kb Kpn I/Hind III fragment (containing pollen/synthetic-cryIA(b)from pCIB4429, the about 4.5 Kb Hind III/Eco RI (PEPC/synthetic-cryIA(b) and the about 2.6 Kb Kpn I/Eco RI fragment from the vector Bluescript.

Other vectors including the pollen promoter/synthetic CryIA(b) chimeric gene include pCIB4428 and pCIB4430. See Figs. 21 and 22. pCIB4430 also contains the PEPC/synthetic-Bt gene described above.

EXAMPLE 47. Production of transgenic maize plants containing the synthetic maize optimized CryIA(b) gene

The example below utilizes Biolistics to introduce DNA coated particles into maize cells, from which transformed plants are generated.

Experiment KC-65

Production of transgenic maize plants expressing the synthetic cryIA(b) gene using a tissue-specific promoter.

Tissue

Immature maize embryos, approximately 1.5-2.5 mm in length, were excised from an ear of genotype 6N615 14-15 days after pollination. The mother plant was grown in the greenhouse. Before excision, the ear was surface sterilized with 20% Clorox for 20 minutes and rinse 3 times with sterile water. Individual embryos were plated scutellum side in a 2 cm square area, 36 embryos to a plate, on the callus initiation medium, 2DG4 + 5 chloramben medium (N6 major salts, B5 minor salts, MS iron, 2% sucrose, with 5 mg/l chloramben, 20 mg/l glucose, and 10 ml G4 additions (Table 1) added after autoclaving.

TABLE 1 - G4 Additions

Ingredient	per liter medium
Casein hydrolysate	0.5 gm
Proline	1.38 gm
Nicotinic acid	.2 mg
Pyridoxine-HCl	.2 mg
Thiamine-HCl	.5 mg
Choline-HCl	.1 mg

Riboflavin	.05 mg
Biotin	.1 mg
Folic acid	.05 mg
Ca pantothenate	.1 mg
p-aminobenzoic acid	.05 mg
B12	.136 μg

Bombardment

Tissue was bombarded using the PDS-1000He Biolistics device. The tissue was placed on the shelf 8 cm below the stopping screen shelf. The tissue was shot one time with the DNA/gold microcarrier solution, 10 μl dried onto the macrocarrier. The stopping screen used was hand punched at ABRU using 10x10 stainless steel mesh screen. Rupture discs of 1550 psi value were used. After bombardment, the embryos were cultured in the dark at 25° C.

Preparation of DNA for delivery

The microcarrier was prepared essentially according to the instructions supplied with the Biolistic device. While vortexing 50 μl 1.0 μ gold microcarrier, added 5 μl pCIB4431 (1.23 μg/μl) (#898) + 2 μl pCIB3064 0.895 μg/μl) (#456) followed by 50 μl 2.5 M $CaCl_2$, then 20 μl 0.1 M spermidine (free base, TC grade). The resulting mixture was vortexed 3 minutes and microfuged for 10 sec. The supernatant was removed and the icrocarriers washed 2 times with 250 μl of 100% EtOH (HPLC grade) by vortexing briefly, centrifuging and removing the supernatant. The microcarriers are resuspended in 65 μl 100% EtOH.

Callus formation

Embryos were transferred to callus initiation medium with 3 mg/l PPT 1 day after bombardment. Embryos were scored for callus initiation at 2 and 3 weeks after bombardment. Any responses were transferred to callus maintenance medium, 2DG4 + 0.5 2,4-D medium with 3 mg/L PPT. Callus maintenance medium is N6 major salts, B5 minor salts, MS iron, 2% sucrose, with 0.5 mg/l 2,4-D, 20 mg/l glucose, and 10 ml G4 additions added after autoclaving.

Embryogenic callus was subcultured every 2 weeks to fresh maintenance medium containing 3 mg/L PPT. All callus was incubated in the dark at 25°C.

The Type I callus formation response was 15%. Every embryo which produced callus was cultured as an individual event giving rise to an individual line.

Regeneration

After 12 weeks on selection, the tissue was removed from callus maintenance edium with PPT and was placed on regeneration medium. Regeneration medium is 0.25MS3S5BA (0.25 mg/l 2,4 D, 5 mg/l BAP, MS salts, 3% sucrose) for 2 weeks followed by subculture to MS3S medium for regeneration of plants. After 4 to 10 weeks, plants were removed and put into GA 7's. Our line KC65 0-6, which became the #176 BT event, produced a total of 38 plants.

Assays

All plants, as they became established in the GA7's, were tested by the chlorophenol red (CR) test for resistance to PPT as described in U.S. Patent Application 07/759,243, filed September 13, 1991, the relevant portions of which are hereby incorporated herein by reference. This assay utilizes a pH sensitive indicator dye to show which cells are growing in the presence of PPT. Cells which grow produce a pH change in the media and turn the indicator yellow (from red). Plants expressing the resistance gene to PPT are easily seen in this test. (#176 = 8 positive/30 negative) Plants positive by the CR test were assayed by PCR for the presence of the synthetic BT gene. (#176 = 5 positive/2 negative/1 dead)

Plants positive by PCR for the syn-BT gene were sent to the phytotron. Once established in the phytotron, they were characterized using insect bioassays and ELISA analysis. Plants were insect bioassayed using a standard European Corn Borer assay (described in Example 5A) in which small pieces of leaf of clipped from a plant and placed in a small petri dish with a number of ECB neonate larvae. Plants are typically assayed at a height of about 6 inches. Plants showing 100% mortality to ECB in this assay are characterized further. ELISA data are shown below. Positive plants are moved to the greenhouse.

Greenhouse/Fertility

Plant number #176-11 was pollinated with wild-type 6N615 pollen. One tassel ear and one ear shoot were produced. All of the embryos from the tassel ear (11) and 56 kernels from Ear 1 were rescued. 294 kernels remained on the ear and dried down naturally.

Pollen from #176-11 was outcrossed to various maize genotypes 5N984, 5NA89, and 3N961. Embryos have been rescued from all 3 outcrosses (5N984 = 45; 5NA89 = 30; 3N961 = 8). Most of the kernels remained on the ears on the plants in the greenhouse and were dried down naturally. DNA was isolated from plant #176-11 using standard techniques and analysed by Southern blot analysis. It was found to contain sequences which hybridize with probes generated from the synthetic cryIA(b) gene and with a probe generated from the PAT gene. These results showed integration of these genes into the genome of maize.

Experiment KC-64

Production of transgenic maize plants expressing the synthetic cryIA(b) gene using a constitutive promoter.

Tissue

Immature maize embryos, approximately 1.5-2.5 mm in length, were excised from an ear of genotype 6N615 14-15 days after pollination. The mother plant was grown in the greenhouse. Before excision, the ear was surface sterilized with 20% Clorox for 20 minutes and rinse 3 times with sterile water. Individual embryos were plated scutellum side in a 2 cm square area, 36 embryos to a plate, on the callus initiation medium, 2DG4 + 5 chloramben medium (N6 major salts, B5 minor salts, MS iron, 2% sucrose, with 5 mg/l chloramben, 20 mg/l glucose, and 10 ml G4 additions Table 1) added after autoclaving.

TABLE 1 - G4 Additions

Ingredient	per liter medium
Casein hydrolysate	0.5 gm
Proline	1.38 gm
Nicotinic acid	.2 mg

Pyridoxine-HCl	.2 mg
Thiamine-HCl	.5 mg
Choline-HCl	.1 mg
Riboflavin	.05 mg
Biotin	.1 mg
Folic acid	.05 mg
Ca pantothenate	.1 mg
p-aminobenzoic acid	.05 mg
B12	.136 μg

Bombardment

Tissue was bombarded using the PDS-1000He Biolistics device. The tissue was placed on the shelf 8 cm below the stopping screen shelf. The tissue was shot one time with the DNA/gold microcarrier solution, 10 μl dried onto the macrocarrier. The stopping screen used was hand punched at ABRU using 10x10 stainless steel mesh screen. Rupture discs of 1550 psi value were used. After bombardment, the embryos were cultured in the dark at 25° C.

Preparation of DNA for delivery

The microcarrier was prepared essentially according to the instructions supplied with the Biolistic device. While vortexing 50 μl 1.0 μ gold microcarrier, added 3.2 μl pCIB4418 (0.85 μg/μl) (#905) + 2 μl pCIB3064 0.895 μg/μl) (#456) + 1.6 μl pCIB3007A (1.7 μg/μl) (#152) followed by 50 μl 2.5 M $CaCl_2$, then 20 μl 0.1 M spermidine (free base, TC grade). The resulting mixture was vortexed 3 minutes and microfuged for 10 sec. The supernatant was removed and the microcarriers washed 2 times with 250 μl of 100% EtOH (HPLC grade) by vortexing briefly, centrifuging and removing the supernatant. The microcarriers are resuspended in 65 μl 100% EtOH.

Callus formation

Embryos were transferred to callus initiation medium with 3 mg/l PPT 1 day after bombardment. Embryos were scored for callus initiation at 2 and 3 weeks after bombardment.

Any responses were transferred to callus maintenance medium, 2DG4 + 0.5 2,4-D medium with 3 mg/L PPT. Callus maintenance medium is N6 major salts, B5 minor salts, MS iron, 2% sucrose, with 0.5 mg/l 2,4-D, 20 mg/l glucose, and 10 ml G4 additions added after utoclaving. Embryogenic callus was subcultured every 2 weeks to fresh maintenance medium containing 3 mg/L PPT. All callus was incubated in the dark at 25°C.

The Type I callus formation response was 18%. Every embryo which produced callus was cultured as an individual event giving rise to an individual line.

Regeneration

After 12 weeks on selection, the tissue was removed from callus maintenance medium with PPT and was placed on regeneration medium and incubated at 25° C using a 16 hour light (50 μE .m-2 . s-1) / 8 hour dark photoperiod. Regeneration medium is 0.25MS3S5BA (0.25 mg/l 2,4 D, 5 mg/l BAP, MS salts, 3% sucrose) for 2 weeks followed by subculture to MS3S medium for regeneration of plants. After 4 to 10 weeks, plants were removed and put into GA 7's. Our line KC64 0-1, which became the #170 BT event, produced 55 plants. Our line KC64 0-7, which became the #171 BT event, produced a total of 33 plants.

Assays

Eleven plants, as they became established in the GA7's, were tested by the chlorophenol red (CR) test for resistance to PPT as per Shillito, et al, above. This assay utilizes a pH sensitive indicator dye to show which cells are growing in the presence of PPT. Cells which grow produce a pH change in the media and turn the indicator yellow (from red). Plants expressing the resistance gene to PPT are easily seen in this test. Plants positive by the CR test were assayed by PCR for the presence of the synthetic BT gene. (Event 170 = 37 positive/18 negative; #171 = 25 positive/8 negative).

Plants positive by PCR for the syn-Bt gene were sent to the phytotron. Once established in the phytotron, they were characterized using insect bioassays and ELISA analysis. Plants were insect bioassayed using a standard European corn borer assay (see below) in which small pieces of leaf of clipped from a lant and placed in a small petri dish with a number of ECB neonate larvae. Plants are typically assayed at a height of about 6 inches. Plants showing 100%

mortality to ECB in this assay are characterized further. ELISA data are shown below. Positive plants are moved to the greenhouse.

Basta screening

Eight of the mature plants from the #170 event were selected for evaluation of Basta (Hoechst) resistance. On one middle leaf per plant, an area approximately 10-14 cm long X the leaf width was painted with 0, 0.4, 1.0 or 2.0% (10 ml of 200 g/L diluted to 100 ml with deionized water) aqueous Basta containing 2 drops of Tween 20/100 ml. Two plants were tested per level. Eight wild-type 6N615 plants of the same approximate age were treated as controls. All plants were observed at 4 and 7 days. All of the control plants eventually died. Throughout the study, none of the #170 plants displayed any damage due to the herbicide.

Pollination

All tassel ears, first ear and, if available, the second ear on the #170 and #171 plants were pollinated with wild-type 6N615 pollen. At least 90% of the plants were female fertile.

Pollen from #171 plants was outcrossed to genotypes 6N615, 5N984, 5NA89, 6F010, 5NA56, 2N217AF, 2NDO1 and 3N961. At least 90% of the plants were shown to be male fertile.

Embryo Rescue

Embryos from the #171 event have been "rescued." Fourteen to 16 days after pollination, the ear tip with 25-50 kernels was cut from the ear with a coping saw. Prior to cutting, the husks were gently peeled away to expose the upper portion of the ear. The cut end of the ear on the plant was painted with Captan fungicide and the husks replaced. The seed remaining on the plant was allowed to dry naturally.

The excised ear piece was surface sterilized with 20% Clorox for 20 minutes and rinsed 3 times with sterile water. Individual embryos were excised and plated scutellum side up on B5 medium (Gamborg) containing 2% sucrose. B5 vitamins are added to the medium after autoclaving. Four embryos were plated per GA7 container and the containers incubated in the dark. When germination occurred, the containers were moved to a light culture room and

incubated at 25° C using a 16 hour light (50 μE .m-2 . s-1) / 8 hour dark photoperiod. The germination frequency is 94%.

Progeny from 15 plants of the #171 event and 2 of the #176 event were rescued using standard embryo rescue techniques and evaluated. All plants were evaluated by insect assay. Plants from the #171 event were also tested in the histochemical GUS assay. In both the insect assay and the GUS assay, the ratio of segregation of the transgenes was 1:1, as expected for a single locus insertion event.

EXAMPLE 48. Analysis of transgenic maize plants

ELISA ASSAY

Detection of cryIA(b) gene expression in transgenic maize is monitored using European corn borer(ECB) insect bioassays and ELISA analysis for a quantitative determination of the level of cryIA(b) protein obtained.

Quantitative determination of cryIA(b) IP in the leaves of transgenic plants was performed using enzyme-linked immunosorbant assays (ELISA) as disclosed in Clark M F, Lister R M, Bar-Joseph M: ELISA Techniques. In: Weissbach A, Weissbach H (eds) Methods in Enzymology 118:742-766, Academic Press, Florida (1986). Immunoaffinity purified polyclonal rabbit and goat antibodies specific for the *B. thuringiensis* subsp. *kurstaki* IP were used to determine ng IP per mg soluble protein from crude extracts of leaf samples. The sensitivity of the double sandwich ELISA is 1-5 ng IP per mg soluble protein using 50 ug of total protein per ELISA microtiter dish well.

Corn extracts were made by grinding leaf tissue in gauze lined plastic bags using a hand held ball-bearing homogenizer (AGDIA, Elkart IN.) in the presence of extraction buffer (50 mM Na_2CO_3 pH 9.5, 100 mM NaCl, 0.05% Triton, 0.05% Tween, 1 mM PMSF and 1 μM leupeptin). Protein determination was performed using the Bio-Rad (Richmond, CA) protein assay.

Using the above procedure, the primary maize transformants described above were analyzed for the presence of cryIA(b) protein using ELISA. These plants varied in height from 6 inches to about three feet at the time of analysis.

Plant	Bt ng/mg soluble protein	5/27/91
176-8	0	0
176-10	700	1585
176-11	760	2195
171-4A	59	
171-6	50	
171-8	60	
171-9	280	
171-13	77	
171-14A	43	
171-14B	60	
171-15	55	
171-16A	13	
171-16B	19	
171-18	19	
176-30	1160	
171-32	980	
171-31	166	
171-30	370	
71-14		
#10 leaf	26	
1 leaf	17	

plant 171-16

#9 leaf 40

#1 leaf 120

EUROPEAN CORN BORER ASSAY

1. One to four 4 cm sections are cut from an extended leaf of a corn plant.

2. Each leaf piece is placed on a moistened filter disc in a 50 X 9 mm petri dish.

3. Five neonate European corn borer larvae are placed on each leaf piece. (Making a total of 5-20 larvae per plant.)

4. The petri dishes are incubated at 29.5 °C.

5. Leaf feeding damage and mortality data are scored at 24, 48, and 72 hours.

EXAMPLE 49. Expression of Bt endotoxin in progeny of transformed maize plants

The transformed maize plants were fully fertile and were crossed with several genotypes of maize. Progeny from these crosses were analyzed for their ability to kill European corn borer (ECB) in a standard ECB bioassay (described immediately above) as well as for the presence of the cryIA(b) protein using ELISA as described above. The ability to kill ECB and the production of cryIA(b) protein correlated. These traits segregated to the progeny with a 1:1 ratio, indicating a single site of insertion for the active copy of the synthetic gene. This 1:1 ratio was true for both the constitutive promoter/synthetic-cryIA(b) plants and the tissue specific promoter/synthetic-cryIA(b) plants (data not shown).

Fig. 23A is a table containing a small subset of the total number of progeny analyzed. This table is representative of a number of different crosses.

Insect assays were done with *Diatrea saccharalis* and *Ostrinia nubilalis* using leaf material (as described above) of transgenic progeny containing a maize optimized CryIA(b) gene. The results of these assays are shown in Fig. 23B. They demonstrate that the maize optimized CryIA(b) gene functions in transformed maize to provide resistance to Sugarcane borer and *Ostrinia nubilalis*.

EXAMPLE 50. Expression Of The CryIA(b) Gene In Maize Pollen

Progeny of the transformed maize plants containing the chimeric pollen promoter/synthetic cryIA(b) gene derived from pCIB4431 were grown in the field to maturity. Pollen was collected and analyzed for the presence of the cryIA(b) protein using standard ELISA techniquesd as described elsewhere. High levels of cryIA(b) protein were detected in the pollen. Progeny from the 35S promoter/synthetic cryIA(b) transformed plant were grown in the greenhouse. Pollen from these plants was analyzed using ELISA, and cryIA(b) protein was detected. Results are shown below in Figure 23C.

It is recognized that factors including selection of plant lines, plant genotypes, synthetic sequences and the like, may also affect expression.

EXAMPLE 51. Expression Of The CryIA(b) Gene Fused To A Pith-Preferred Promoter.

pCIB4433 (Fig. 36) is a plasmid containing the maize optimized CryIA(b) gene fused with the pith-preferred promoter isolated from maize. This plasmid was constructed using a three-way ligation consisting of:

1) pCIB4418, cut with BstEII and BamHI; 1.8 Kb fragment

2) pBtin1, cut with NsiI and BstEII; 5.9 Kb fragment; pBtin1 is described elsewhere in this application

3) PCR fragment VI-151 was generated in a PCR reaction using standard conditions as described elsewhere in this application.

PCR primers utilized were:

KE150A28: 5'-ATT CGC ATG CAT GTT TCA TTA TC-3' (SEQ ID NO:93)

KE151A28: 5' - GCT GGT ACC ACG GAT CCG TCG CTT CTG TGC AAC AAC C-3' (SEQ ID NO:94)

After the PCR reaction, the DNA was checked on an agarose gel to make sure the reaction had proceeded properly. DNA was recovered from the PCR reaction using standard conditions described elsewhere and subsequently cut with the restriction enzymes NsiI and BamHI using standard condition. After cutting, the fragment was run on a 2% NuSieve gel and the desired band recovered as described elsewhere. The DNA was used in the ligation described above.

After ligation (under standard condition), the DNA was transformed into competent *E. coli* cell.

Transformation was carried out using microprojectile bombardment essentially as described elsewhere in this application. Embryos were transferred to medium containing 102μg/ml PPT 24 hours after microprojectile bombardment. Resulting callus was transferred to medium containing 40 μg/ml PPT after four weeks. Plants were regenerated without selection.

A small sample of plants (3-5) was assayed by PCR for each event. Further codes were added to indicate different positions and distances of embryos with respect to the microprojectile bombardment device. Plants were sent to the greenhouse having the following codes:

JS21A TOP	Plants B.t. PCR Positive
JS21A MID	Plants B.t. PCR Positive
JS21C BOT	Plants B.t. PCR Positive
JS22D MID	Plants B.t. PCR Positive
JS23B MID	Plants B.t. PCR Negative (for control)

Leaf samples from the regenerated plants were bioassayed for insecticidal activity against European corn borer as described in Example 48 with the results shown in Fig. 23D.

ELISA analysis of leaf samples to quantify the level of CryIA(b) protein expressed in the leaves was carried out as described in Example 48 with the results shown in Fig. 23E.

DEPOSITS

The following plasmids have been deposited with the Agricultural Research Culture Collection (NRRL)(1818 N. University St., Peoria, IL 61604) under the provisions of the Budapest Treaty: pCIB4418, pCIB4420, pCIB4429, pCIB4431, pCIB4433, pCIB5601, pCIB3166 and pCIB3171.

* * * * * * * * * * *

The present invention has been described with reference to specific embodiments thereof; however it will be appreciated that numerous variations, modifications, and embodiments are possible. Accordingly, all such variations, modifications and embodiments are to be regarded as being within the spirit and scope of the present invention.